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STUDIES ON THE TOXICITY OF DICLOFENAC SODIUM IN BROILER BIRDS

MVSc THESIS

By

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COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY
ANJORA, DURG
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STUDIES ON THE TOXICITY OF THE DICLOFENAC SODIUM IN BROILER BIRDS

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Ms. Teenu Jain

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CERTIFICATE - I

This is to certify that the thesis entitled **"Studies on the toxicity of diclofenac sodium in broiler birds"** submitted in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.) is a record of the bonafide research work carried out by **Ku. Teenu Jain** under my guidance and supervision. The subject of the thesis has been approved by the student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or has been published / published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by her.

Date: November 19, 2007

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This is to certify that the thesis entitled **"Studies on the toxicity of diclofenac sodium in broiler birds"** submitted by Ku.Teenu **Jain** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.) in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science** in the Department of Veterinary Pharmacology and Toxicology has been approved by the Student's Advisory Committee in collaboration with External Examiner.

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LIST OF SYMBOLS/ABBREVIATIONS

Abbreviation	Full Form
%	Per cent
@	At the rate of
µ	Micron
µl	Microlitre
^{0}C	Degree Centigrade
ATP	Adenosine triphosphate
DCP	Dicalcium phosphate
DLC	Differntial leucocyte count
H & E	Heamatoxylin and Eosin
g/dl	Gram/deciliter
GPT	Glutamic pyruvic transaminase
IFCC	International Federation of Clinical Biochemistry
LDH	Lactate dehydrogenase
LSP	Lime stone powder
Min	Minute
Ml	Mililitre
NAD	Nicotinamide adenosyl dinucleotide
Nm	Nanometer
NSAIDs	Non steroidal Anti-inflammatory Drugs
P	Probability
Po	Per os
RT	Room Temperature
SE	Standard Error
Sec	Second
Std	Standard
TEC	Total Erythrocyte Count
TLC	Total Leucocyte Count
U/L	Unit/Litre

CHAPTER - I

INTRODUCTION

Like humans, animals also experience adverse effects when treated with medicinal products. All drugs have side effects. While the side effects of an approved drug on humans or the target animal may be mild, its effect on wildlife, other animals and birds could be far more deleterious. These effects may be related to differences in species or pharmacological or toxicological properties of the substance used.

Diclofenac was one of the extensively prescribed nonsteroidal anti-inflammatory drugs (NSAIDs) in veterinary medicine used to treat a wide range of painful and inflammatory conditions. Diclofenac owes its pharmacological action to preferential inhibition of cyclooxygenase enzyme (COX I) and the consequent block in prostaglandin synthesis (Rainsford, 2006). Its widespread use in animal health has been linked to near extinction of vultures in the Indian subcontinent, and as such the drug is withdrawn from use in the year 2006. Vulture deaths were due to severe renal damage causing visceral gout following scavenging on dead livestock treated shortly before death (Oaks *et al.,* 2004). The hepatotoxic and nephrotoxic effects of diclofenac sodium in both human and experimental animals have also been reported (Tolman, 1998; Castel *et al.,* 1997; Dunk *et al.,* 1982; Sergio *et al.,* 1997).

The etiopathology of diclofenac-induced visceral gout in birds is not understood properly. Toxic effects on the kidney of birds have been observed following treatment with a number of NSAIDs (Oaks *et al.,* 2004; Anderson *et al.,* 2005). Since the NSAIDs have no accepted therapeutic application in birds, their pharmacology was not investigated. But a wide variety of indications exist for

which treatment with NSAIDs could be beneficial (Bauck, 1990) in bird medicine. The examples include trauma (Baert, 2003), coccidiosis (Vermeulen, 2002), sudden death syndrome or broiler ascites (Balog *et al.,* 2000), heat stress (Oliver and Birrenkott, 1981; Stillborn *et al.,* 1998), locomotion disturbances (Danbury *et al*.,1997; Hocking *et al*.,1997) and pain related to beak trimming (Glatz *et al*., 1992).

The unanticipated vulture deaths due to indirect exposure of later have necessitated detail studies of diclofenac in the genesis of visceral gout in the avian species. It could be argued that with the reduction in vulture numbers other scavenger birds could also be susceptible to diclofenac toxicity. Moreover animal byproducts used in poultry feed (bone meal, mutton tallow and blood meal) containing diclofenac residues may also have adverse effects on health of the birds. The status of diclofenac and its toxicity on other scavenger birds like crow is not known. Though, there is a lot of interspecies differences exist, the present experimental investigations could throw some light on the toxicity of diclofenac sodium, if any to broiler birds. Therefore, the present study was considered worthwhile to assess whether diclofenac is similarly toxic to broiler birds as in vultures. The experimental investigations are planned with the following objectives:

1. To evaluate the toxicity, if any, with single oral dose of diclofenac sodium (mammalian dose and a higher dose) in broiler birds.

2. To evaluate the toxicity, if any, with repeated oral mammalian doses of diclofenac sodium in broiler birds.

3. To evaluate certain hematological, biochemical and pathological alterations, following treatment with diclofenac sodium in broiler birds as per above schedule.

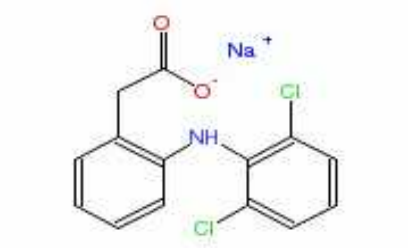
CHAPTER - II

REVIEW OF LITERATURE

DRUG: DICLOFENAC SODIUM

Chemistry

Diclofenac is a phenyl acetic acid derivative with the following chemical structure.



Sodium {2-[(2, 6-dichlorophenyl) amino] phenyl} acetate

M Wt: 318.13

PHARMACOLOGY

Diclofenac is a phenyl acetic acid derivative that possesses analgesic, antipyretic and anti-inflammatory activities. It is an inhibitor of cyclooxygenases and thus inhibits prostaglandin synthesis. It is rapidly and completely absorbed after oral administration and peak concentration in plasma is reached within 2 to 3 hr. It is extensively bound to plasma proteins (99%), and its half-life in plasma is 1 to 2 hrs. It is metabolized in liver to 4- hydroxylated forms; after sulfation, the metabolites are excreted in the urine (65%) and bile (35%) (Hardman *et al.*, 2001).

TOXICOLOGY

Kappus (1986) and Braun *et al*. (1993) reported that diclofenac caused a rare but potentially fatal hepatotoxicity that might be associated with the formation of reactive metabolites.

Diclofenac associated renal failure has been reported in humans with prolonged exposure (Murray and Brater, 1993) or with preexisting renal disease (Davies and Anderson, 1997) but these reports did not describe renal pathology.

. The damaging effect of NSAIDs on mammalian kidney is most commonly due to their effect on renal vasculature and blood supply (Murray and Brater, 1993).

Kertz-Romell *et al*. (1993) reported that the damage in hepatocytes induced by diclofenac sodium was associated with an idiosyncratic reaction.

Mitochondrial damage and NADPH deficiency are thought to be responsible for diclofenac sodium hepatotoxicity (Farrel, 1997).

The miscellaneous effects associated with the use of NSAIDs include hepatotoxicity, aseptic meningitis, diarrhoea and central nervous system depression (Boothe, 1989).

Moreno-Sanchez *et al.* (1999) studied the effects of anti-inflammatory drug diclofenac sodium on mitochondrial respiration, ATP synthesis and membrane potential. The drug was found to stimulate ATP synthesis and collapsed membrane potential in mitochondria. Diclofenac also blocked the activity of the adenine nucleotide translocase and inhibited mitochondrial ATPase activity.

Diclofenac is more toxic to drug metabolizing cells than to non enzyme metabolizing cell lines. Despite of fact that diclofenac itself was effective in impairing ATP synthesis by mitochondria, there was evidence that toxicity was also related to the drug metabolism and was reduced by the addition of cytochrome P-450 inhibitors to the culture medium (Bort *et al.*, 1999).

Bort *et al*. (1999) investigated the acute effects of diclofenac sodium on the viability and functionality of cultured hepatocytes to discriminate between possible

mechanisms of toxicity and found that a decrease in ATP levels prelude cell death. They found evidence that toxicity was also related to drug metabolism.

Renal toxicities mainly caused by NSAID are due to inhibition of COX I enzyme. These include renal vasoconstriction and renal insufficiency. This nephropathy does not occur frequently in domestic animals**,** but patients suffering from cardiac, liver or renal diseases, hypovolumic patients and patients receiving nephrotoxic drugs are predisposed (Boothe, 2001).

Aydin *et al*. (2002) investigated the effect of different doses of diclofenac sodium on liver and renal tissues. Forty albino rats weighing 200-220 g were divided equally into four groups. The rats in control group were each injected intramuscularly with 1 cc of physiological saline. The other three groups were given diclofenac sodium doses. The rats in the first, second and third groups were intramuscularly injected with diclofenac sodium at a low, medium and high dose of 50, 100 and 150 mg/Kg live weight/day, respectively, every day for five days. At the end of the experimental period, the animals were sacrificed, autopsied and liver and kidney tissue samples were prepared for histopathological assessment. No significant ($p > 0.05$) change were observed in the liver and kidney tissues of control group. The diclofenac sodium treatment significantly ($p < 0.001$) affected the histology of both liver and kidney. Histopathological changes in the liver sections stained with hematoxylin and eosin in all diclofenac groups included cloudy swelling and hydropic degeneration of liver cells, focal sinusoidal and vena centralis dilatation, proliferation of bile ducts in the portal areas, enlargement of the periportal area with mononuclear cell infiltration, hyperemia and dose dependent fibrous tissues proliferation and focal necrosis. Cloudy swelling and hydropic degeneration were seen in the tubular epithelial cell of kidney tissue of all

diclofenac sodium treated groups. Necrosis, peritubular lymphocyte infiltration, stromal fibrous tissue proliferation and hyperemia were observed in the second and third groups. In the liver and kidney tissue of third group which was given a high dose of diclofenac sodium, necrosis cloudy swelling and hydropic degeneration and inflammation were rather wide spread and intensive as compared to the group given a low dose. The increase in fibrous tissue in the kidney and liver that cause irregularities in the periportal areas was only seen in the group given a high dose. These results suggest that a high dose of diclofenac sodium causes meaningful changes in the liver and kidney tissues.

Ramesh, *et al.* (2002) investigated the toxicity of diclofenac sodium in dogs. Eight mongrel dogs were used in the study. Diclofenac sodium (Voveran, 3mg/kg, po) was administered twice daily for 4 days. Blood samples were collected at o, 6, 12, 24, 48, 72 and 96hr after the administration of first dose for the estimation of serum biochemical parameters. BUN and creatinine levels were significantly increased, but the levels of AST, ALT and ALP were not altered significantly. PM examination revealed ulcerations of the stomach of dog treated with diclofenac. Histopathology showed distension of renal tubules. No histopathologic changes were observed in the liver.

Masubuchi *et al*. (2003) studied the role of mitochondrial permeability transition (MPT) in the pathogenesis of diclofenac-induced hepatocyte injury by using isolated mitochondria and primary culture hepatocytes from rats. Incubation of energized mitochondria with succinate in the presence of Ca^{2+} and diclofenac resulted in mitochondrial swelling, leakage of accumulated Ca^{2+}, membrane depolarization, and oxidation of nicotinamide adenine dinucleotide phosphate and protein thiol. All of these phenomena were suppressed by coincubation of the

mitochondria with cyclosporin A, a typical inhibitor of MPT, showing that diclofenac opened the MPT pore. It was also suggested that reactive oxygen species probably generated during mitochondrial respiration and/or voltage-dependent mechanism was involved in MPT, which are proposed as mechanisms of MPT by uncouplers of mitochondrial oxidative phosphorylation. Culture of hepatocytes for 24 hr with diclofenac caused a decrease in cellular ATP, leakage of lactate dehydrogenase and membrane depolarization. The hepatocyte toxicity thus observed was attenuated by coincubation of the hepatocytes with cyclosporin A and verapamil, a Ca^{2+} channel blocker. In conclusion, these results showed the important role of MPT in pathogenesis of hepatocyte injury induced by diclofenac and its possible contribution to human idiosyncratic hepatotoxicity.

Vermeulen (2002) conducted a trial with ibuprofen (NSAID) at an oral dose of 100mg/kg and observed a significant reduction in oocyst shedding and intestinal coccidial lesion.

Arun and Azeez (2004) carried out post mortem examination of vulture carcasses died due to diclofenac toxicity and revealed visceral gout as the cause of mortality in most of the dead birds.

Hallare *et al*. (2004) reported one of the most frequently detected pharmaceuticals in environmental water samples, the anti-rheumatic drug, diclofenac. Despite its increasing environmental significance, investigations concerning the effects of this drug on the early developmental stages of aquatic species are lacking up to now. So to determine the developmental toxicity and proteotoxicity of diclofenac sodium on the growing fish embryos, they exposed eggs of zebrafish to six concentrations of diclofenac (0, 1, 20, 100, 500, 1000, and 2000 μg /L) using DMSO as solvent. Early life stage parameters such as egg and

embryo mortality, gastrulation, somite formation, movement and tail detachment, pigmentation, heart beat, and hatching success were noted and described within 48 and 96 hrs of exposure. After the 96 hr exposure, the levels of stress proteins (hsp 70) were determined in both the diclofenac-treated and respective DMSO controls. Results showed no significant inhibition in the normal development until the end of 96 hrs for all exposure groups. However, there was a delay in the hatching time among embryos exposed to 1000 and 2000 µg /L. Late-hatched embryos (108 hr) did not differ morphologically from normally hatched embryos. The mortality and average heart rate data did not show significant differences for all embryos in both diclofenac-treated and DMSO control groups. No significant malformations were likewise noted among all developing embryos throughout the exposure period. The levels of heat shock proteins in diclofenac-treated and control embryos did not differ significantly. DMSO control embryos, on the other hand, showed a concentration-dependent increase in hsp 70 levels. They suggested possible modulating effect of diclofenac in DMSO-triggered expression of stress proteins and this might have a possible repercussion on the use of DMSO as solvent in any toxicity assay. Since the present data indicate no significant embryotoxicity and proteotoxicity induced by diclofenac and due to the fact that the concentrations of diclofenac used in the present study is up to 2000-fold higher than the concentrations detected in the environment, it is unlikely that this drug would pose a hazard to early-life stages of zebra fish.

Oaks *et al*. (*2004*) carried out gross post mortem examination of 259 adult and sub adult Oriental White Backed Vultures (OWBVs) from 2000-2002. They reported that 85% had grossly apparent urate deposition on the surface of visceral organ organs characteristic of visceral gout. They found direct correlation between

residues of anti-inflammatory drug diclofenac with renal failure due to gout**.** In all the Oriental White Backed Vultures with visceral gout, the only significant histopathological lesion noted by the others was severe acute renal tubular necrosis and uric acid crystals formation in the kidney and other tissues. Diclofenac residue and renal disease were reproduced experimentally in OWBVs by direct oral exposure and through feeding vultures diclofenac treated livestock. To verify the renal toxicity of diclofenac in OWBVs, they administered single dose of veterinary diclofenac to four captive, non-releasable juvenile OWBVs. Two were given mammalian level dose of 2.5 mg/kg (high dose) and the other two 0.25 mg/kg (low dose). Both of high and one of the low dose vultures died as a result of renal failure and visceral gout within 38 to 56 hr after administration. Plasma sample collected one hour after administration for one high dose and one low doses of vultures indicated normal uric acid levels but 24 hr after administration, both vultures had developed hyperuriceamia. Diclofenac residue concentrations of 0.29, 1.1 and 0.16 µg per gm were present in the kidney of two high dose vultures and one low dose vulture that died as a result of renal failure. Although the other low doses vultures developed hyperuriceamia, it remained normal and did not have microscopic lesions and detectable diclofenac residue at necropsy. To verify that carcasses of treated livestock contained sufficient diclofenac concentration to cause renal failure and death in the vultures that scavenge upon that, ten juvenile OWBVs were fed meat from a buffalo or goat that was injected intramuscularly with 2.5 mg/kg veterinary diclofenac once daily for three days and that were slaughtered 4h after the last injection and resulting diclofenac residue concentration in the buffalo and goat kidney, liver and muscle were 5.7, 1.5 and 0.76 µg per gm respectively and goat kidney, liver and muscle were 0.94, 0.22 and 0.19 µg per gm respectively. Eight

OWBVs received dose of 0.005 mg/kg two of these vultures died from renal failure at 4 to 6 days after exposure. At necropsy, they had residue concentration of 0.07 and 0.38 µg per gm in the kidney. The combined mortality rate of 13 out of 20 (65%) in the exposed vultures and 0 out of 6 (0%) in the control group vulture indicated highly significant relationship between renal failure and exposure to diclofenac.

Shultz *et al*. (2004) reported that veterinary use of diclofenac sodium could likely to be the major cause of visceral gout in vultures. Seventy two percent of the bird examined at the post mortem had extensive visceral gout. Diclofenac was detected at concentration in the range 0.004-0.16µg/gm in liver and kidney of affected vultures. Seventy seven per cent (10/13) of the Oriental White Backed vultures and 63 per cent (5/8) of the Long Billed vultures had detectable residue of diclofenac. There was complete and highly significant association between the presence of diclofenac and that of visceral gout. All fourteen birds with gout had detectable diclofenac, but diclofenac was not found in the four birds without gout. A high proportion of dead vultures had visceral gout and that gout was strongly associated with diclofenac residues.

Gajera and Prajapati (2005) reported that feeding of diet with diclofenac @ 2.5, 5, 10, 15 ppm for 15 days resulted in mortality of broilers due to visceral gout between 5 and 11 days of experiment at levels 5, 10 and 15 ppm; whereas, no mortality was seen in 2.5 ppm group.

Meteyer *et al*. (2005) proposed pathophysiology of diclofenac poisoning in free living and experimentally exposed Oriental White Backed vultures (*Gyps bengalensis*) and reported severe acute necrosis of proximal convoluted tubules. Glomeruli, distal convoluted tubules were relatively spared in vultures that had

early lesions. In most vultures, however lesions became extensive with large urate aggregates obscuring renal architecture. Inflammation was minimal. Extensive urate deposition on the surface and within organ parenchyma (visceral gout) was consistent in vultures with renal failure.

Very little is known about the physiologic effect of NSAIDs in birds. Research in mammals has shown that diclofenac inhibits formation of prostaglandins. Blood flow to the avian kidney is very different from that of mammals. The renal portal system via the afferent renal portal vein, is the primary nutrient source for the avian renal cortex and does not supply the renal medulla or medullary cone (Braun, 1993; Goldstein and Skadhaughe, 2000; Smith *et al.*, 2000). They proposed that diclofenac induced renal failure in the OWBVs is through the inhibition of the modulating effect of prostaglandin on angiotensin II mediated adrenergic stimulation. Renal portal valve opens in response to adrenergic stimulation, redirecting portal blood to the caudal venacava and bypassing the kidney. If diclofenac removes the modulating effect of prostaglandins on the renal portal valves, indiscriminate action of these valves would redirect the primary nutrient blood supply away from the renal cortex resulting in ischemic necrosis of the cortical proximal convoluted tubules.

Ramírez-Alcántara *et al.* (2005) investigated the influence of cholestyramine co-treatment on diclofenac enteropathy in fed rats and diclofenac gastropathy in fasted rats. Male Sprague-Dawley rats were gavaged with diclofenac (50 mg/kg) plus cholestyramine (50 mg/kg), or an equivalent amount of Flotac, a 1:1 wt/wt mixture of diclofenac and cholestyramine presently used in human therapeutics in Mexico. Ulceration of the GI tract, serum proteins, and bile salts were assessed at 3, 12, or 24 hr. Cholestyramine alone produced no detectable alterations in small

intestinal integrity or serum bile salts levels. Fed rats given Flotac or diclofenac plus cholestyramine showed patterns of small intestinal ulceration and serum protein decline that were similar to rats given only diclofenac. Thus, no influence on enteropathy was detected. In contrast, fasted rats given Flotac showed a modest reduction in the number and length of gastric lesions compared to rats given diclofenac. The observed attenuation of gastric lesions with Flotac is consistent with a role for direct cytotoxicity in NSAID gastropathy.

Swan *et al*. (2005) tested the toxicity of diclofenac to a Eurasian (*Gyps fulvus*) and an African (*Gyps Africanus*) species, neither of which threatened. A dose of 0.8mg/kg of diclofenac was highly toxic to both species, indicating that they are at least as sensitive to diclofenac as *G.bengalensis*, for which they estimated an LD_{50} of 0.1-0.2mg/Kg.They suggested that diclofenac is likely to be toxic to all eight Gyps species, and that *G. africanus*, which is phylogenetically close to *G. bengalensis*, would be a suitable surrogate for the safety testing of alternative drugs to diclofenac.

Green *et al*. (2006) reported that veterinary use of the non-steroidal anti-inflammatory drug, diclofenac, appears to be a major cause of the vulture population decline. Vultures are likely to be exposed to the drug when they feed on carcasses of livestock that were treated with diclofenac before death. They measured the concentration of diclofenac in the tissues of treated Indian humped and European cattle (*Bos indicus* and *Bos taurus*) in relation to the interval between dosing and death. They used a dose-response model to assess the risk posed to wild vultures if they feed on carcasses of treated livestock. Diclofenac concentrations in fat, intestine, kidney and liver were considerably higher than those in muscle, but concentrations in the first four tissues initially depleted more rapidly (half-life 6-8

hr) with time since the last injection of the drug, compared to muscle (half-life 15 hr). Depletion rates became much slower in all tissues 25-98 h after the last injection. Diclofenac concentration, averaged across the carcass, was enough to cause appreciable mortality (> 10% of birds per meal) if oriental white-backed vultures *G. bengalensis* were to take a large meal from the carcass of an animal that was given its last dose of the drug within a day or two before death. Vultures that feed selectively on tissues with high concentrations of the drug, such as kidney, liver and intestine, would be exposed to a higher risk and for longer after dosing. The tissues of cattle treated with diclofenac are a hazard to wild vultures that feed on an animal that dies within a few days after treatment. Intestine, kidney and liver have the highest diclofenac concentrations, but the concentration averaged across all the edible tissues of the carcass is also hazardous. They recommended withdrawal of diclofenac from veterinary use on animals whose carcasses may become available to scavenging vultures. In *ex situ* and *in situ* conservation projects, vultures should be fed on carcasses of animals that are known not to have been treated with diclofenac in the week before death.

Manov *et al.* (2006) reported that scarce side effects of diclofenac is associated with rare but sometimes fatal hepatotoxicity characterized by delayed onset of symptoms and lack of a clear dose-response relationship. The toxicity has consequently been categorized as metabolic idiosyncrasy. In fact, the acyl glucuronide of the drug has been demonstrated to be reactive and capable of covalent modification of cellular proteins, binding covalently to liver proteins in rats depending on the activity of multidrug resistance protein 2, a hepatic canalicular transporter. Both oxidative stress (caused by putative diclofenac cation radicals or nitroxide and quinone immune-related redox cycling) and mitochondrial injury

(protonophoretic activity and opening of the permeability transition pore) alone or in combination, have been implicated in diclofenac toxicity. In some cases, immune-mediated liver injury is involved, as inferred from inadvertent rechallenge data and from a number of experiments demonstrating T cell sensitization. Caspases 8 and 9 are apparently active caspases in diclofenac-induced apoptosis. In addition, an early dose-dependent increase of Bcl-X_L expression parallel to the generation of reactive oxygen species in the mitochondria was found. In conclusion, the mitochondrial pathway is very likely the only pathway involved in diclofenac-induced apoptosis, which is related to CYP-mediated metabolism of diclofenac and the highest apoptotic effect is produced by the metabolite 5OH-diclofenac. To date, cumulative damage to mitochondrial targets seems a plausible putative mechanism to explain the delayed onset of liver failure, perhaps even superimposed on an underlying silent mitochondrial abnormality. Although Gomez-Lechon *et al.* (2003) have demonstrated that diclofenac induces apoptosis by alteration of mitochondrial function and generation of reactive oxygen species (ROS), liver injury could not be reproduced in current animal models. Nevertheless, it is noteworthy that ultrastructural damage was found in the liver of rainbow trout exposed to various concentrations of diclofenac, thus illustrating differences in reactivity between mammals and other vertebrates.

Prakash Reddy *et al*. (2006) compared Nimesulide with diclofenac sodium toxicity in poultry. In this study Vanaraja and PB1 birds of 6 weeks old (either sex) were mixed and equally divided into 5 groups of 10 birds each. Each bird was inoculated with nimesulide @5mg and 2mg and diclofenac sodium @5mg per kg body weight basis. All the groups were observed for a period of 28 days. 40% mortality was observed within a 12 days in diclofenac treated group while birds

inoculated with nimesulide remained normal. No significant differences in the weight gain, hematology, total protein content among nimesulide and diclofenac treated groups (survived birds) were observed when compared with control group. Serum creatinine, cholesterol and alkaline phosphatase in nimesulide treated group were comparable ($p > 0.05$) to control groups. Nimesulide treated group also did not show any histopathological lesions; whereas diclofenac treated birds showed histopathological lesions in liver and kidney.

Swan *et al*. (2006) administered meloxicam to 35 captive *Gyps* vultures and found no apparent ill effects. They also undertook a phased programme of safety testing of meloxicam on the African white-backed vulture *Gyps africanus,* which was previously established to be as susceptible to diclofenac poisoning as the endangered Asian *Gyps* vultures. They estimated the likely maximum level of exposure (MLE) of wild vultures and dosed birds by gavage (oral administration) with increasing quantities of the drug until the likely MLE was exceeded in a sample of 40 *G. africanus*. Subsequently, six *G. africanus* were fed tissues from cattle which had been treated with a higher than standard veterinary course of meloxicam prior to death. In the final phase, ten Asian vultures of two of the endangered species *(Gyps bengalensis*, *Gyps indicus)* were dosed with meloxicam by gavage; five of them at more than the likely MLE dosage. All meloxicam-treated birds survived all treatments, and none suffered any obvious clinical effects. Serum uric acid concentrations remained within the normal limits throughout, and were significantly lower than those from birds treated with diclofenac in other studies. We conclude that meloxicam is of low toxicity to *Gyps* vultures and that its use in place of diclofenac would reduce vulture mortality substantially in the Indian subcontinent.

Cuthbert, *et al.* (2007) reported that Veterinary treatment of livestock with diclofenac, a non-steroidal anti-inflammatory drug (NSAID), has caused catastrophic declines of *Gyps* vultures in Asia. This has highlighted a lack of knowledge on the potential impacts of NSAIDs on scavenging birds. Surveys of veterinarians and zoos document the outcomes of the treatment of over 870 scavenging birds from 79 species. As well as diclofenac, carprofen and flunixin were associated with mortality, with deaths observed in 13 and 30 per cent of cases, respectively. Mortality was also found following treatment with ibuprofen and phenylbutazone. NSAID toxicity was reported for raptors, storks, cranes and owls, suggesting that the potential conservation impact of NSAIDs may extend beyond *Gyps* vultures and could be significant for New World vultures. In contrast, there were no reported mortalities for the NSAID meloxicam, which was administered to over 700 birds from 60 species. The relative safety of meloxicam supports other studies indicating the suitability of this NSAID to replace diclofenac in Asia.

Hong, *et al.* (2007) investigated the expression levels of cytochrome P450 1A, p53 and vitellogenin in three different tissues of male medaka fish after exposure to diclofenac that is one of the main concerns among pharmaceuticals frequently found in sewage treatment plant (STP) effluents. The results showed that cytochrome P450 1A, p53 and vitellogenin were highly expressed in tissue-specific gene expression patterns after exposure to 8 mg/L and 1 μg/L of diclofenac. These elevated expression levels of three biomarkers suggested that diclofenac has potential to cause cellular toxicity, p53-related genotoxicity and estrogenic effects. It is also noteworthy that diclofenac has the potential to cause these effects even at an environmentally relevant concentration of 1 μg/L.

Patel, *et al*. (2007) conducted an experiment to know the role of marginal high protein (27%), high calcium (1.6%), water deprivation and diclofenac drug (2.5mg/kg/day for 15 days) for causation of visceral gout in broiler chicks. Broiler chicks were divided into different groups with different treatments and samples were collected on 3rd, 6th, 9th, 12th and 15th days of age from each group. They found that oral administration of diclofenac caused hyperuricemia and mortality due to visceral gout in broiler chicks. The mean level of plasma uric acid on 12th and 15th day collection were 19.04 ± 5.39 and 23.45 ± 4.56 respectively and were significantly higher as compared to respective collection of control group.

Triebskorn *et al.* (2007) in order to assess potential effects of human pharmaceuticals in aquatic wildlife, conducted laboratory experiments with carbamazepine, clofibric acid, metoprolol, and diclofenac using fish as test organisms. For each substance, at least one environmentally relevant concentration was tested. In liver, kidney, and gills of trout and carp exposed to carbamazepine, clofibric acid, metoprolol and diclofenac. Ultrastructural effects were qualitatively described and semi-quantitatively assessed. The obtained assessment values were compared with previously published data for diclofenac-induced effects in rainbow trout tissues. Quantitative analyses of protein accumulated in kidneys of diclofenac-exposed trout corroborated previously published data which indicated that diclofenac induced a severe glomerulonephritis resulting in a hyaline droplet degeneration of proximal kidney tubules. The investigations provided information on the general health status of the pharmaceutical-exposed fish, and allowed a differential diagnosis of harmful effects caused by these human pharmaceuticals in non-target species. For the different cytological effects observed, lowest observed effect concentration (LOEC) for at least three of the test substances (diclofenac,

carbamazepine, metoprolol) were in the range of environmentally relevant concentrations (1 µg/L).

CHAPTER - III

MATERIALS AND METHODS

Experiments were conducted to evaluate the single and repeated dose oral toxicity of diclofenac sodium in broiler birds. The various materials and methods employed are described in this Chapter.

EXPERIMENTAL BIRDS

Healthy adult broiler birds around six weeks old (Vencob) obtained from M/S Indian Broiler Group, Rajnandgaon (CG) for the present experiment. All the birds were kept under deep litter system of management and had free access to standard broiler ration and drinking water. The balanced ration was composed of Maize (59.7%), Soya (26.3%), Fish Meal (5%), DCP (1.3%), LSP (.7%), Salt (.25%), Soda (.25%) and premix (1%).

For the present investigations diclofenac procured from the market (Voveran® tablets containing 50 mg diclofenac sodium IP of Novartis India Limited, Pune) was used. The tablets were powdered and administered in distilled water in suspended form.

EXPERIMENTS

PHASE-I: Single Dose Oral Toxicity Study

Eighteen adult broiler birds were divided at random into three groups of six birds each. Prior to administration of the test material the birds were weighed individually and were fasted overnight. First group (Group I) received distilled water orally and served as control. Second group received diclofenac sodium at the rate of 2 mg/kg body weight (mammalian therapeutic dose) and the third group received diclofenac sodium @ 20 mg/kg body weight (high dose). The

administration of drug was carried out orally by gavage using a specially designed gastric needle attached to a syringe in volume not exceeding 3 ml per bird. The experimental design is given in Table 1.

Table 1: Design of Single Dose Oral Toxicity Study

Group No.	No. of Birds	Treatment	Period of study
I	6	Control (vehicle)	7days
II	6	Diclofenac sodium @ 2mg/kg (mammalian therapeutic dose)	7days
III	6	Diclofenac sodium @ 20 mg/Kg	7days

General Observation:

Immediately after the administration of drug/test material, birds were placed in deep litter system and they were carefully and constantly observed for behavioral changes, toxicity signs and symptoms (time of onset, nature and severity) and mortality if any up to a period of seven days.

Biochemical Parameters:

To estimate the below listed parameters approximately 2 ml of blood was drawn from the wing/tarsal/jugular vein from each bird with the help of a 22 gauze needle at pre treatment (0 hr) and at 12, 24 and 36 hr of post treatment period into heparinized vials for the separation of plasma. Heparinization was done with 1% heparin solution and 0.1 ml was sufficient to moisten the inside of the 5 ml vial. Plasma samples were stored in the deep freeze at -20^{o}C until the estimations were completed. The estimations were carried out with the help of different Bayer's diagnostic reagent kits using ROBONIK'S Semi-autoanalyzer, as per the standard methods given in kits.

Total Protein: For the purpose of total protein analysis, Total Protein Kit (Biuret Method), manufactured by Bayer's Diagnostics India Ltd. was used.

Principle: Peptide bond from protein formed a blue-violet coloured complex with cupric ions in an alkaline medium. The intensity of colour was proportional to the number of bond formed and the colour was read at 540 nm (530-570). The final colour was stable for 8 hrs. Total protein was expressed as g/dl.

Procedure: Reagent reconstitution was done by adding 41ml of distilled water to one bottle of Reagent 1 (Biuret reagent) and then contents of Reagent 1A (Surfactant) was added to it. The samples and reconstituted reagents were brought to room temperature before use. The instrument was set using these general system parameters; Reaction type: Endpoint, Reaction slope: increasing, Wavelenghth: 546nm (530-570nm), Flowcell Temperature: 30^{o}C, Incubation: 20 Min. RT, Sample Volume: 10µl, Reagent Volume: 1ml, Std. Concentration: 6g/dL and Zero Setting: with reagent blank.

Dispensed into test tube:

	Blank	Standard	Test
Reconstituted reagent	1ml	1ml	1ml
Standard	-	10 µl	-
Sample	-	-	10 µl

Incubated for 20 minutes at room temperature, mixed and read.

Serum Albumin: Serum albumin was estimated by BCG method using Bayer's diagnostic kit. The results were expressed in g/dL.

Principle: Albumin in a buffered solution reacts with the anionic bromocresol green (BCG) with a dye binding reaction to give a proportionate green colour which is measured at 628nm (600-650 nm). The final colour is stable for 10 min.

Procedure: The samples and the Reagent 1 (Bromocresol Green) was brought to room temperature prior to use. The instrument was set using these general system

parameters; Reaction type: Endpoint, Reaction slope: increasing, Wavelenghth: 628 nm (600-650nm), Flowcell Temperature: 30^{o}C, Incubation: 1 Min. RT, Sample Volume: 10µl, Reagent Volume: 1ml, Std. Concentration: 5g/dL and Zero Setting: with reagent blank.

Dispensed into test tube:

	Blank	Standard	Test
Reconstituted reagent	1ml	1ml	1ml
Standard	-	10 µl	-
Sample	-	-	10 µl

Incubated for 1 minute at room temperature, mixed and read.

Uric acid: For the purpose of uric acid analysis, uric acid kit (Enzymatic Method), manufactured by Bayer's Diagnostics India Ltd. was used.

Principle: Uric acid was converted by uricase into allantoin and hydrogen peroxide which in presence of peroxidase (POD) oxidizes the chromogen into a red colored compound which was read at 500nm (492-550). The final color of reaction was stable for 15 minutes. Uric acid was expressed as mg/dl.

$$\text{Uric acid} + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrin} + \text{DHBS} \xrightarrow{\text{POD}} \text{Red quinone} + H_2O + HCl$$

DHBS = 3,5-Dichloro-2-Hydroxybenzene Sulfonic Acid

Procedure: Reagent reconstitution was done by adding one bottle of Reagent 1(Enzyme/Cromogen) to one bottle of Reagent 1A (Buffer). The samples and reconstituted reagents were brought to room temperature before use. The instrument was set using these general system parameters, Reaction type: Endpoint, Reaction slope: increasing, Wavelenghth: 510nm (492-550), Flowcell Temperature: 30^{o}C, Incubation: 5min at 37^{o}C, Sample Volume: 25µl, Reagent Volume: 1ml, Std. Concentration: 6g/dL and Zero Setting: with reagent blank

Dispensed into test tube:

	Blank	Standard	Test
Reconstituted reagent	1ml	1ml	1ml
Standard	-	25 µl	-
Sample	-	-	25 µl

Incubated for 5 minutes at 37^{o}C, mixed and read.

Creatinine: For the purpose of Creatinine analysis, Creatinine kit (Picrate Method), manufactured by Bayer's Diagnostics India Ltd. was used.

Principle: Creatinine in alkaline medium reacted with Picrate and formed an orange colored compound. Under the specific conditions of the assay, the rate of development of color was proportional to the concentration of creatinine in the sample when measured at 500nm (490-510). Creatinine was expressed in mg/dl.

Procedure: The working solution was prepared by mixing equal volume of Reagent 1 (Picrate) to Reagent 2 (Sodium Hydroxide) in a clean beaker. The samples and working solution were brought to room temperature before use. The instrument was set using these general system parameters, Reaction type: Fixed type, Reaction slope: increasing, Wavelenghth: 500nm (490-510nm), Flowcell Temperature: 25 ^{o}C, 30 ^{o}C, or 37^{o}C, Delay time: 30 sec, No. of readings: 2, Interval: 120sec, Sample Volume: 100µl, Reagent Volume: 1ml, Path length: 1cm and Zero Setting: with distilled water.

Calibration

Dispensed into test tube:

	Standard
Working Solution	1mL
Sample	100 µL

Mixed and read immediately for factor calculation.

TEST

Dispensed into test tube:

	Test
Working Solution	1mL
Sample	100 µL

Mixed and read immediately.

Plasma Glutamic Pyruvic Transaminase (PGPT): For the purpose of PGPT analysis, PGPT (ALT) kit (UV Kinetic (IFCC) Method), manufactured by Bayer's Diagnostics India Ltd. was used.

Principle:

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightleftharpoons{\text{GPT}} \text{L-Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightleftharpoons{\text{LDH}} \text{L-Lactate} + NAD^{+}$$

There was decrease in the absorption at 340 nm as NADH was converted to NAD. The rate of decrease in absorbance was measured and was promotional to SGPT activity in the sample. The ALT was expressed in U/L.

Procedure: Reagent reconstitution was done by adding one bottle of Reagent 1 (Enzyme) to one bottle of Reagent 1A (Buffer). The samples and reconstituted reagents were brought to room temperature before use. The instrument was set using these general system parameters, Reaction type: Kinetic, Reaction slope: decreasing, Wavelenghth: 340nm, Flowcell Temperature: 37^{o}C, Delay time: 60sec, No. of readings: 3, Read time: 60 sec, Sample Volume: 100µl, Reagent Volume: 1ml, Path length: 1cm, Factor: 1746 and Zero Setting: with distilled water.

Dispensed into test tube:

	Test
Reconstituted Reagent	1mL
Sample	100µL,

Pathological Observations:

Gross pathology: All the live birds were sacrificed at the end of observation period (on 8th day) and dead ones were autopsied and were examined for the appearance of lesions if any. Samples of tissues from different organs (showing lesions) were collected in 10% formal saline for histopathological examinations.

Murexide Test: The Murexide test mentioned by Sharma (1997), was performed, where the white chalky material was taken from the serosal surface of visceral organs of the died birds due to diclofenac toxicity. This was mixed with nitric acid and dried over a flame. A drop of concentrated ammonia was added, a purplish red (mauve) colour development indicated presence of uric acid.

Histopathology: Formal saline fixed tissues from different organs after washing in running tap water were dehydrated in sequences of acetone, cleared in sequences of xyline and embedded in Paraffin wax. Paraffin blocks were prepared, sections were cut at 2-3µ and stained with hematoxylin and eosin as per the standard methods in Singh and Sulochana (1997).

PHASE–II: Repeated dose oral toxicity study

Twelve adult broiler birds were divided at random into two groups of 6 birds each. First group (Group I) received distilled water orally and served as control. Second group received diclofenac sodium at the rate of 2 mg/kg, po, (mammalian therapeutic dose) daily for 14 days. The administration of drug was carried out orally by gavage using a specially designed gastric needle attached to a syringe in volume not exceeding 1 ml per bird. The design of repeated dose toxicity experiment is shown Table 2.

Table 2: Design of Repeated Dose Oral Toxicity Study

Group No.	No. of birds	Treatment	Period of study
I	6	Control (vehicle)	14 days
II	6	Diclofenac sodium @2mg/kg body weight (mammalian therapeutic dose) daily for 14 days.	14 days

General Observations

Immediately after the administration of drug / test material, birds were placed in deep litter system and they were carefully and constantly observed for behavioral changes, toxicity signs and symptoms (time of onset, nature and severity), body weight and mortality if any up to a period of fourteen days.

Biochemical parameters:

To estimate these parameters approximately 2 ml of blood was drawn from the tarsal/jugular vein from each bird with the help of a 22 gauze needle at pre treatment (0 day) and after the completion of treatment period (15^{th} day) into heparinized vials for the separation of plasma. Heparinization was done with 1% heparin solution, at which concentration 0.1 ml was sufficient to moisten the inside of the 5 ml vial. Plasma sample was stored in the deep freeze at $-20^{o}C$ until the estimations were completed. Parameters were estimated with the help of different Bayer's diagnostic reagent kits using ROBONIK'S Semi-autoanalyzer, as per the standard methods given in kits. The biochemical parameters viz. total protein, albumin, glutamic pyruvate transaminase, uric acid and creatinine were estimated as in case of single dose oral toxicity

Hematological Parameters:

To estimate the following parameters approximately 2ml of blood was drawn from the tarsal/jugular vein from each bird with the help of 22 gauze needle at 0 day and 14 day post-treatment of the observation period and was collected in heparinized vials. Heparinization was done with 1% heparin solution, at which concentration 0.1 ml was sufficient to moisten the inside of the 5 ml vial. Estimations were done by standard methods as described by Jain (1986).

Hemoglobin: Hemoglobin concentration was estimated by Sahli's Heamometer. In the presence of N/10 HCl, the heam part of the hemoglobin got lysed and reacted with acid in acidic medium. As a result a brown coloured complex, acid hematin was formed. That was compared with Comparater and results were expressed in g percentage.

Total Erythrocyte Count (TEC): Erythrocytes were counted with the help of Heamocytometer in Neubauer's counting chamber using Nett and Herrick's fluid as a diluting fluid and the results were expressed in millions per cubic mm.

Total Leukocyte count (TLC): TLC was done with the help of Heamocytometer in Neubauer's counting chamber using Nett and Herrick's fluid as a diluting fluid and the results were expressed in thousands per cubic mm.

Differential Leukocyte Count (DLC): Blood smears were prepared immediately after collection of samples, stained with Leishman's stain and then counted and the results were expressed in percentage.

Clotting Time: Clotting time was calculated with help of Capillary Method. Clotting time is the oozing of blood to formation of clot i.e. fibrin mesh formation. A prick was made in the superficial vein of bird. Blood was allowed to rise in

capillary tube by capillary action and the time was noted. After that in every 30 seconds the capillary tube was broken until some thread was observed during breaking and time was noted.

Pathological Observations:

Gross Pathology: All birds were sacrificed at the end of observation period (on 15th day) and were examined for the appearance of lesions if any. Samples of tissues from different organs (showing lesions) were collected in 10% formal saline for histopathological examinations.

Histopathology: Formal saline fixed tissues from different organs after washing in running tap water were dehydrated in sequences of acetone, cleared in sequences of xyline and embedded in Paraffin wax. Paraffin blocks were prepared and sections were cut at 2-3µ and stained with hematoxylin and eosin as per the standard methods in Singh and Sulochana (1997).

STATISTICAL ANALYSIS

The data of treatment groups at different observation intervals were analyzed by ANOVA to find out statistical variation between the mean values of different groups at different intervals of the observation period using the software SPSS 10 for Windows.

CHAPTER - IV

RESULTS AND DISCUSSION

The present investigations included studies on the toxicity of diclofenac sodium in broiler birds. The various observations and results are presented and discussed in this Chapter.

GENERAL OBSERVATIONS

Two groups (II and III) of six broiler birds each, were orally administered with a single dose of the test drug, diclofenac sodium @ 2 mg/kg and 20 mg/kg respectively. The birds in group II did not show any toxicity sign or symptom except diarrheic faeces in some birds. In Group III, three birds out of the six showed segregatory behaviour, dullness, depression, lethargy and disinclination to feed and water. Subsequently, these three birds died due to toxicity within 36 hr of post treatment. The other three birds were otherwise normal except that they had diarrheic blood tinged faeces all through the observation period. None of the control birds showed any signs of toxicity.

The birds which received repeated oral dose of diclofenac sodium @ 2 mg/kg/day for 14 consecutive days did not reveal any visible adverse reaction indicative of diclofenac toxicity, except loose droppings. The control birds of this group remained healthy all through the experimental period.

No data are available on acute toxicity of diclofenac in poultry birds.

Prakash Reddy *et al*. (2006) also observed 40 % mortality in Vanraja and PB1 birds within 12 days following inoculation of diclofenac sodium @ 5mg/kg. Swan *et al*. (2005) also reported that a dose of 0.8 mg/kg of diclofenac sodium was highly toxic to *Gyps fulvus* and *Gyps africanus* species of vultures and found the oral LD_{50} of diclofenac in *Gyps bengalensis* as 0.1 to 0.2 mg/kg.

In present study a single dose of 20 mg/kg, po, caused 50 % mortality in adult broiler birds. The results indicate that diclofenac is also toxic to adult broiler birds but they are less sensitive as compared to reports in vultures.

The data of body weight of birds in control and drug-treated groups are shown in Table 3. The mean post-treatment body weight (on 15th day) in the two groups were (2023.33 ± 91.78 and 2748.33 ± 104.09 gm) as against their respective body weights at pre-treatment (1493.33 ± 34.80 and 1487.50 ± 30.04 gm). The variation in the pre- and post-treatment mean body weight between the two groups were not statistically significant. The mean gain in body weight in control and diclofenac treated groups were 530 ± 86.37 and 555 ± 94.78 gm, respectively.

Table 3: Effect of repeated dose oral administration of diclofenac sodium on body weight in broiler birds

Group No.	Treatment	Mean Body Weight (g) ± SE		Gain in Body Weight
		Pre-treatment	Post-treatment	
I	Control	1493.33^{a} ± 34.80 (6)	2023.33^{b} ± 91.78 (6)	530 ± 86.37
II	Diclofenac sodium @2 mg/kg/day for 14 days	1487.5^{a} ± 30.04 (6)	2748.33^{b} ± 104.09 (6)	555 ± 94.78

Figures in parentheses refer to number of observations
Mean values at pre- and post-treatments with similar superscripts are not significantly different ($p > 0.05$)

BIOCHEMICAL PROFILE

The effect of oral administration of diclofenac sodium at single or repeated doses on various blood biochemical parameters of broiler birds, such as plasma total protein, albumin, uric acid, creatinine and glutamic pryruvic transaminase (PGPT) was investigated. In the single dose study, the pre-treatment normal values were

determined from the blood at 0 hr and the post treatment values were quantified from the blood collected at 12, 24, and 36 hr post treatment.

Effect on Plasma Total Protein

Table 4 and Fig. 1 explain the effect of single low dose (@ 2 mg/kg, po) and high dose (20 mg/kg, po) of diclofenac sodium on total protein in broiler birds. The mean total protein levels in the control, low dose and high dose groups at pre-treatment ranged from 4.83 ± 0.33 to 4.93 ± 0.06 g/dl and were statistically similar. In the control group, the levels at three post treatment intervals were also statistically similar (4.55 ± 0.14 to 4.88 ± 0.15 g/dl). Following administration of single low dose of diclofenac sodium @ 2mg/kg (GroupII), total protein decreased significantly at 12hr (1.93 ± 0.08 g/dl) and 24hr (2.68 ± 0.02 g/dl) post-treatment as compared to pre-treatment. However, the protein levels in this group at 36 hr post-treatment returned to near pre-treatment value. In Group III, the surviving birds had significantly lowered protein level at 12 to 36 hr post-treatment (1.66 ± 0.02 to 3.97 ± 0.25 g/dl) as compared to that at pre-treatment. The dead birds in Group III also showed significant reduction at 12 and 24 hr post treatment (1.07 ± 0.03 to 1.66 ± 0.08 g/dl), as compared to that of pre-treatment. The levels in both low and high dose groups were significantly decreased at 12 and 24hr of post-treatment. Moreover the decrease in levels at 24 hr post-treatment in the dead birds of group III was significantly higher than the birds in group II and alive birds of group III. However, at 36 hr post treatment levels returned to control level in group II and live birds of group III.

Table 4: Effect of single dose oral administration of diclofenac sodium on total protein in broiler birds

Group No.	Dose of Diclofenac Sodium		Mean Plasma Total Protein (g/dl) ± SE			
			Pre-treatment (0hr)	Post-treatment (hr)		
				12	24	36
I	Control		$4.88^{a,c}$ ± 0.15 (6)	4.55^{a} ± 0.14 (6)	4.75^{a} ± 0.20 (6)	$4.88^{a,f}$ ± 0.15 (6)
II	2 mg/kg		$4.88^{b,c}$ ± 0.21 (6)	1.93^{d} ± 0.08 (6)	2.68^{e} ± 0.20 (6)	$4.16^{b,f}$ ± 0.20 (6)
III	20 mg/kg	Live birds	4.83^{c} ± 0.33 (3)	1.66^{d} ± 0.02 (3)	3.11^{e} ± 0.06 (3)	3.97^{f} ± 0.25 (3)
		Dead birds	4.93^{c} ± 0.06 (3)	1.66^{d} ± 0.08 (3)	1.07 ± 0.03 (3)	Died

Figures in parentheses refer to number of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 1: Plasma total protein levels of broiler birds treated with single oral dose of diclofenac sodium

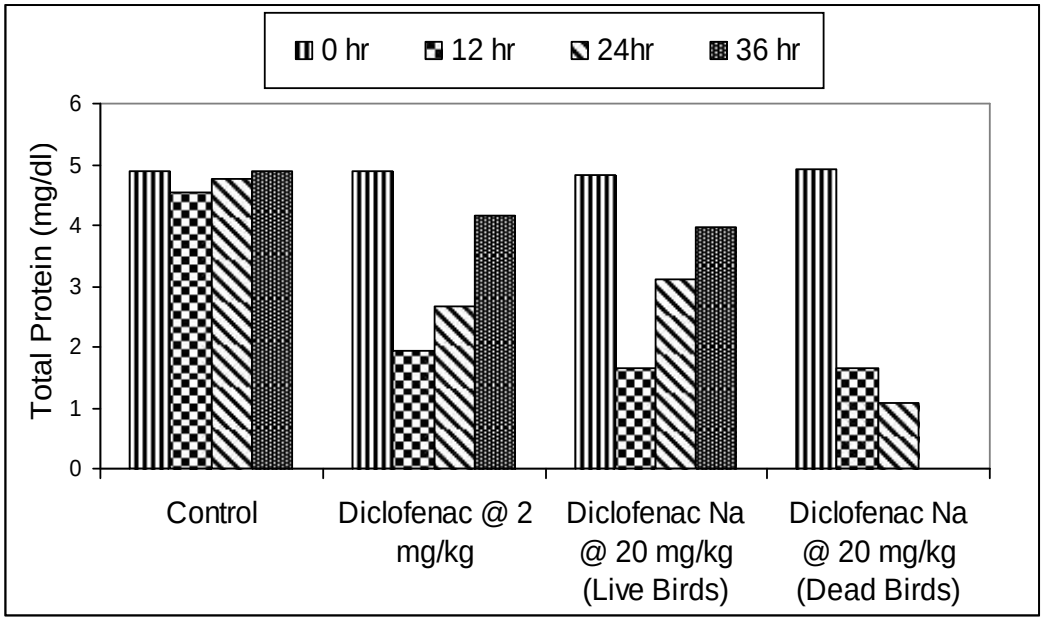


Table 5 shows the mean plasma total protein levels of repeated dose study at pre- and post-treatment in the control birds and those treated with repeated dose of diclofenac sodium @ 2 mg/kg/day, po (Group II). In the control group of this study, the mean plasma total protein levels at pre- and post-treatment were 4.53 ± 0.26

and 4.70 ± 0.21 g/dl respectively, and were statistically identical. Following repeated administration of diclofenac sodium the proteins levels were not statistically altered (4.35 ± 0.27 to 4.52 ± 0.28 g/dl).

Table 5: Effect of repeated oral administration of diclofenac sodium on plasma total protein levels in broiler bird

Group No.	Treatment	Mean Total Protein (g/dl) ± SE	
		Pre-treatment	Post-treatment
I	Control	4.53 ± 0.26 (6)	4.70 ± 0.21 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	4.52 ± 0.28 (6)	4.35 ± 0.27 (6)

Figures in parentheses refer to number of observation

Effect on Plasma Albumin

Mean plasma albumin levels in broiler birds in the three groups of single dose study are presented in the Table 6 and Fig. 2. The levels at pre-treatment in the three groups were statistically similar (2.07 ± 0.16 to 2.40 ± 0.05g/dl). The three post-treatment values of the control group (2.34 ± 0.15 to 2.70 ± 0.09 g/dl) were also statistically similar to that of pre-treatment. In the low dose group, albumin levels were 1.64 ± 0.08, 1.97 ± 0.02 and 2.23 ± 0.01 g/dl at 12, 24 and 36 hr of post treatment respectively, where the 12 and 24hr post-treatment values significantly lowered from the pre-treatment. However, the albumin levels returned to near pre-treatment level at 36 hr of post-treatment. In the high dose group, the birds which survived up to the end of observation period showed the albumin levels in the range of 1.29 ± 0.08 to 2.45 ± 0.08 g/dl during post-treatment, where the level was significantly decreased at 12 and 24 hr as against pre-treatment level. Among the birds which died due to diclofenac toxicity the levels at 12 and 24 hr post-treatment

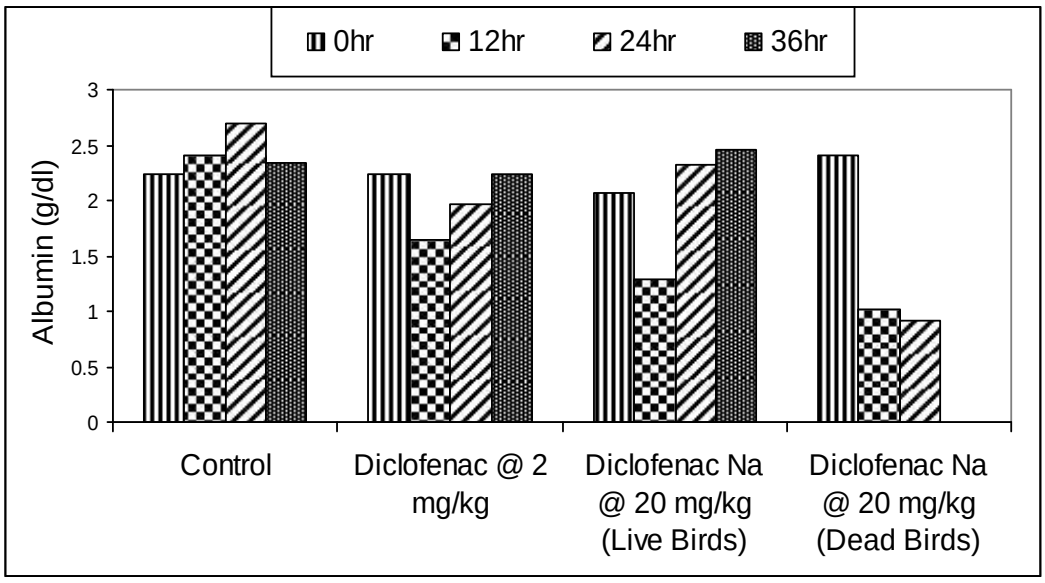
were 1.01 ± 0.01 and 0.91 ± 0.03 g/dl respectively, which were significantly lower than pre-treatment. The depression at plasma albumin levels at 24 hr post-treatment in the dead birds of group III was significantly more than the birds of Group II and the live birds of Group III. However, the levels also returned to control level at 36 hr post-treatment in both Group II and live birds of Group III.

Table 6: Effect of single dose oral administration of diclofenac sodium on plasma albumin in broiler birds

Group No.	Dose of Diclofenac sodium		Mean Plasma Albumin (g/dl) ± SE			
			Pre-treatment	Post-treatment (hr)		
				12	24	36
I	Control		$2.23^{a,f}$ ±0 .01 (6)	2.40^{a} ± 0.13 (6)	$2.70^{a,h}$ ± 0.09 (6)	$2.34^{a,i}$ ± 0.15 (6)
II	2 mg/kg		$2.23^{b,f}$ ± 0.01 (6)	1.64^{c} ± 0.08 (6)	$1.97^{c,h}$ ± 0.02 (6)	$2.23^{b,i}$ ± 0.01 (6)
III	20 mg/kg	Live Birds	$2.07^{d,f}$ ± 0.16 (3)	$1.29^{d,g}$ ± 0.08 (3)	$2.33^{d,h}$ ± 0.17 (3)	$2.45^{d,i}$ ± 0.08 (3)
		Dead Birds	2.40^{f} ± 0.05 (3)	$1.01^{e,g}$ ± 0.01 (3)	0.91^{e} ± 0.03 (3)	Died

Figures in parentheses refer to no. of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 2: Plasma albumin levels of broiler birds treated with single oral dose of diclofenac sodium



The mean albumin values following repeated oral administration of diclofenac sodium @ 2mg/kg/day for consecutive 14 days are presented in the Table 7. Pre- and post-treatment albumin values in the repeated dose group (2.23 ± 0.08 and 2.06 ± 0.07g/dl) were statistically similar to that of control group (2.23 ± 0.07 to 2.25 ± 0.06g/dl).

Table 7: Effect of repeated oral administration of diclofenac sodium on plasma albumin levels in broiler birds

Group No.	Treatment	Mean Albumin (g/dl) ± SE	
		Pre-treatment	Post-treatment
I	Control	2.23 ± 0.07 (6)	2.25 ± 0.06 (6)
II	Diclofenac sodium @2 mg/kg/day	2.23 ± 0.08 (6)	2.06 ± 0.07 (6)

Figures in parentheses refer to no. of observations

The result showed that high dose of diclofenac sodium caused significant decrease in the total protein and albumin. Decrease in both is related to hepatic damage (Lumeij, 1999). The high dose of diclofenac sodium has been reported to cause significant change in liver and kidney tissue in rats (Aydin *et al*. 2002). In the present study also, high dose of diclofenac in broiler birds lead to development of hepatotoxicity and nephrotoxocity as evidenced by increased levels of PGPT, uric acid and creatinine with consistent histopathological changes in the above organs. Therefore, the observed decrease in total protein and albumin might be attributed to the liver and kidney damage

Effect on Plasma Uric Acid

The mean plasma uric acid levels in the three groups at different intervals are presented in the Table 8 and Fig. 3. The levels during pre-treatment and at all the three

post-treatment intervals were statistically similar in control group, which ranged from 6.18 ± 0.62 to 6.58 ± 0.56 mg/dl. Following oral administration of diclofenac sodium @ 2mg/kg, the plasma uric acid levels at 12, 24 and 36 hr post treatment were 19.81± 0.41, 11.03 ± 0.99 and 7.43 ± 0.68 mg/dl respectively, where the 12 and 24 hr uric acid level were significantly higher than the pre-treatment level. The level then declined and returned back to normal at 36 hr.

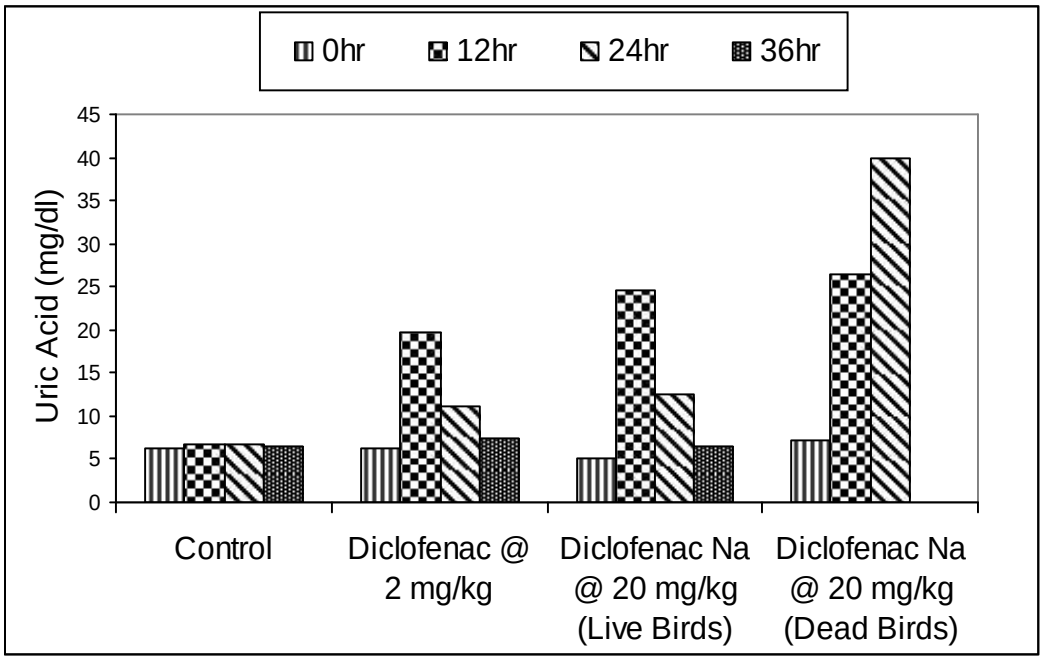
Following oral administration of diclofenac sodium at high dose (20mg/kg) in Group III, the plasma uric acid levels among the surviving birds at 12, 24 and 36 hr of post treatment were 24.50 ± 0.73, 12.60 ± 0.49 and 6.50 ± 0.27 mg/dl respectively, where the 12 and 24 hr levels were significantly higher than those of pre-treatment and 36 hr post-treatment. Further, the level at 12 hr post-treatment in Group II and live birds of Group III were significantly higher than that of 24 and 36 hr post treatment levels. Whereas in the dead birds of Group III, the level at 24 hr was significantly much higher than that of 12 hrs level. In dead birds of Group III, the levels were significantly higher at 12 and 24 hr of post-treatment (26.43 ± 0.93 to 39.93 ± 1.79 mg/dl) as compared to that at pre-treatment. The birds of both Groups II and III showed significantly elevated uric acid levels at 12 and 24 hr of post treatment as compared to those in control group. The increase in uric acid levels at 12 and 24 hr post-treatment in the dead birds of Group III were significantly higher than the birds of Group II and surviving birds of Group III. The value of uric acid in birds of Group II and live birds of Group III at 36 hr of post-treatment was not statistically significant indicating the return of uric acid level to the control level by 36 hr.

Table 8: Effect of single dose oral administration of diclofenac sodium on plasma uric acid levels in broiler birds

Group No.	Dose of Diclofenac sodium		Mean Plasma Uric Acid (mg/dl) ± SE			
			Pre-treatment (0hr)	Post-treatment (hr)		
				12	24	36
I	Control		$6.18^{a,d}$ ± 0.62 (6)	6.66^{a} ± 0.58 (6)	6.68^{a} ± 0.45 (6)	$6.58^{a,f}$ ± 0.56 (6)
II	2 mg/kg		$6.18^{b,d}$ ± 0.62 (6)	19.81 ± 0.41 (6)	11.03^{e} ± 0.99 (6)	$7.43^{b,f}$ ± 0.68 (6)
III	20 mg/kg	Live birds	$5.06^{c,d}$ ± 0.18 (3)	24.50 ± 0.73 (3)	12.60^{e} ± 0.49 (3)	$6.50^{c,f}$ ± 0.27 (3)
		Dead birds	7.3^{d} ± 0.55 (3)	26.43 ± 0.93 (3)	39.93 ± 1.79 (3)	Died

Figures in parentheses refer to no. of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 3: Plasma uric acid levels of broiler birds treated with single oral dose of diclofenac sodium



The mean plasma uric acid levels following repeated oral administration of diclofenac sodium @ 2 mg/kg/day for 14 days are depicted in the Table 9 and Fig. 4. The uric acid levels at pre- and post-treatment period in the control (5.81 ± 0.30 and 5.66 ± 0.03mg/dl) and at pre-treatment level in the Group II (5.76 ± 0.36) are

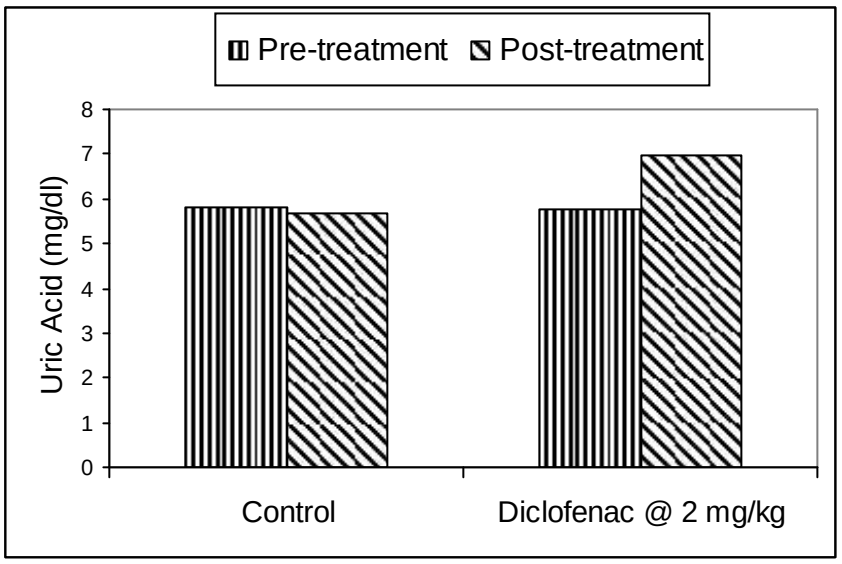
statistically similar. However at post-treatment, the uric acid level (6.97 ± 0.32) was significantly higher than that of control group.

Table 9: Effect of repeated oral administration of diclofenac sodium on plasma uric acid levels in broiler birds

Group No.	Treatment	Mean Uric Acid (mg/dl) ± SE	
		Pre-treatment	Post-treatment
I	Control	$5.81^{a,b}$ ± 0.30 (6)	5.66^{a} ± 0.03 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	5.76^{b} ± 0.36 (6)	6.97 ± 0.32 (6)

Figures in parentheses refer to number of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 4: Plasma uric acid levels of broiler birds treated with repeated oral dose of diclofenac sodium (2 mg/kg/day)



Effect on Plasma Creatinine

Table 10 and Fig. 5 illustrates the effect of single low and high doses of diclofenac sodium on plasma creatinine in broiler birds. The mean plasma creatinine levels in control group (I) at pre-treatment and at different post-treatment intervals

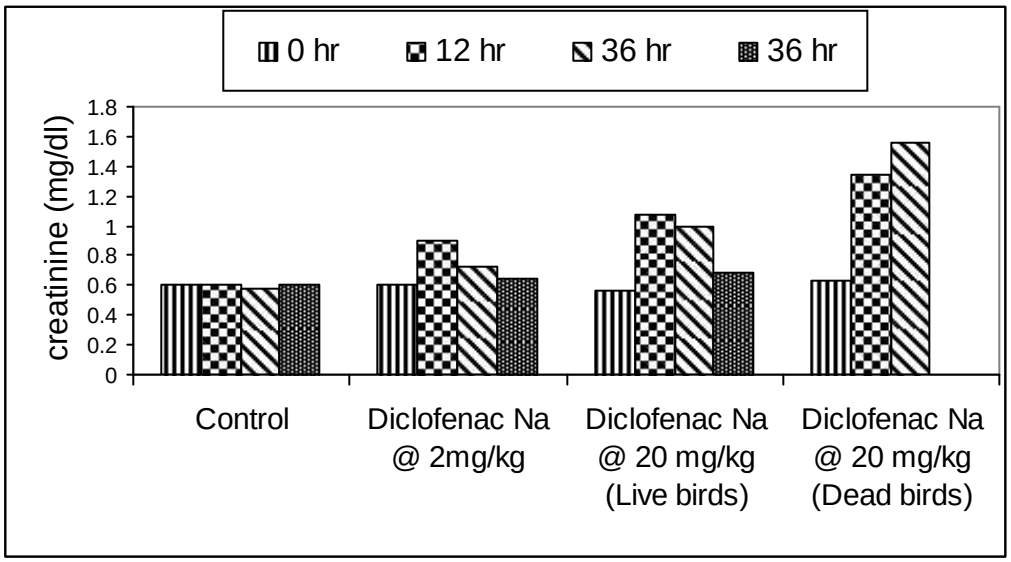
varied from 0.58 ± 0.03 to 0.61 ± 0.02 mg/dl with no statistically significant variation. In Group II (2 mg/kg) the creatinine levels at pre-treatment and 12, 24 and 36 hr of post-treatment intervals were 0.60 ± 0.04, 0.90 ± 0.03, 0.73 ± 0.01 and 0.64 ± 0.02 mg/dl respectively, where the 12 and 24 hr post-treatment values were significantly elevated. However, the creatinine level subsequently returned to pre-treatment level at 36 hr post-treatment. In case of live birds (Group III) the creatinine levels during pre-treatment and at 12, 24 and 36 hr of post-treatment were 0.56 ± 0.08, 1.08 ± 0.04, 0.99 ± 0.00 and 0.69 ± 0.01 mg/dl respectively, where the12 and 24 hr post-treatment levels remained significantly elevated as compared to pre-treatment level. In the same group, the birds which died after 24 hr showed the creatinine levels as 1.35 ± 0.12 and 1.56 ± 0.03 mg/dl at 12 and 24hr post-treatment, which were significantly higher than the pre-treatment level. The 36 hr post-treatment levels, in all the birds of group II and live birds of group III were statistically similar indicating the return of creatinine level in these groups by 36 hr. The increase in levels at 12 and 24 hr post-treatment in the dead birds of Group III were significantly much higher than the birds of Group II and live birds of Group III.

Table 10: Effect of single dose oral administration of diclofenac sodium on creatinine in broiler birds

Group No.	Dose of Diclofenac sodium		Mean Plasma Creatinine (mg/dl) ± SE			
			Pre-treatment (0hr)	Post-treatment (hr)		
				12	24	36
I	Control		$0.60^{a,f}$ ± 0.04 (6)	0.60^{a} ± 0.03 (6)	0.58^{a} ± 0.03 (6)	$0.61^{a,g}$ ± 0.02 (6)
II	2 mg/kg		$0.60^{b,f}$ ± .04 (6)	0.90 ± .03 (6)	0.73^{c} ± 0.01(6)	$0.64^{b,c,g}$ ± 0.02 (6)
III	20 mg/kg	Live Birds	$0.56^{d,f}$ ± 0.08 (3)	1.08^{e} ± 0.04 (3)	0.99^{e} ± 0.00 (3)	$0.69^{d,g}$ ± 0.01 (3)
		Dead birds	0.63^{f} ± 0.03 (3)	1.35 ± 0.12 (3)	1.56 ± 0.03 (3)	Died

Figures in parentheses refer to no. of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 5: Plasma creatinine levels of broiler birds treated with single oral dose of diclofenac sodium



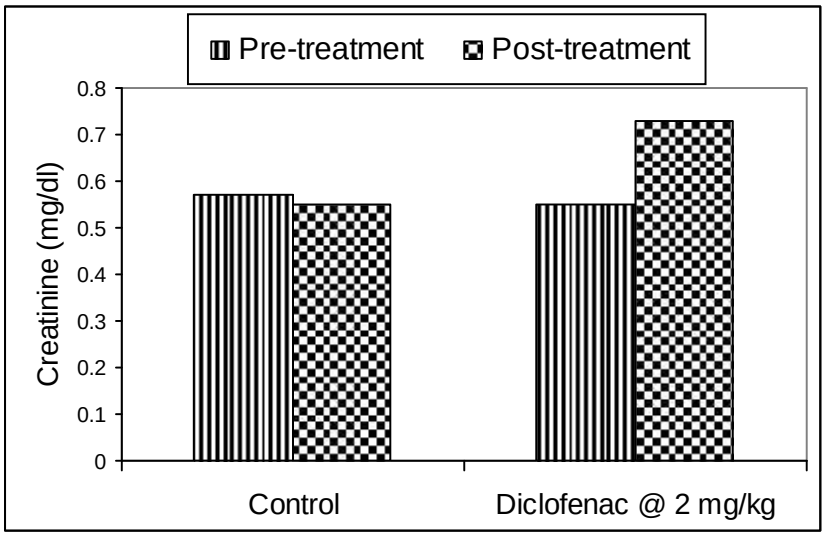
The mean creatinine levels at pre- and post-treatment of repeated dose groups are explained in the Table 11 and Fig. 6. The creatinine levels at pre- and post-treatment period in the control birds (0.57± 0.03 and 0.55 ± 0.02 mg/dl) and at pre-treatment interval in diclofenac sodium treated group (0.55 ± 0.04 mg/dl) were statistically similar. However at post-treatment period these birds had significantly higher (0.73 ± 0.04 mg/kg) than that of control group.

Table 11: Effect of repeated oral administration of diclofenac sodium on plasma creatinine levels in broiler birds

Group No.	Treatment	Mean Creatinine (mg/dl) ± SE	
		Pre-treatment	Post-treatment
I	Control	$0.57^{a,b}$ ± 0.03 (6)	0.55^{a} ± 0.02 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	0.55^{b} ± 0.04 (6)	0.73 ± 0.04 (6)

Figures in parentheses refer to no. of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 6: Plasma creatinine levels of broiler birds treated with repeated oral dose of diclofenac sodium (2 mg/kg/day)



Studies of Uma *et al.* (1999) reported clinical gout in chicken when serum uric acid level exceeded 25.5 mg/dl as compared to 4.2 to 6.4 mg/dl in the control birds and those of Prajapati (2003) revealed that blood uric acid level in normal birds ranged from 4.5 to 5 mg/dl and when its levels were beyond 47 mg/dl the birds started dieing with typical visceral gout, which are in close concurrence with the present results.

Lin *et al*. (1976) reported the normal blood values of uric acid in broilers and layers chicken as 4.85 and 4.87 mg/dl respectively and did not notice any clinical signs with blood uric acid values below 8.27mg/dl; whereas slight clinical signs were evident with values of 8.27 to 21.4 mg/dl and severe signs leading to death were evident with values over 47mg/dl. These observations also support the results of the present study. Several other workers also reported increased levels of uric acid in blood of birds with gout (Mishra *et al*., 1980; Pathak, 2001). Swan *et al*. (2005) found increased level of uric acid as high as 140 and 160 mg/dl, 12 and 24 hr after

administration of diclofenac @ 0.8mg/kg orally in *Gyps Africanus* vultures.

Oaks *et al*. (2004) also recorded highly elevated blood uric acid levels corresponding to 65.4 and 77.5 mg/dl at 24 hr after administration of diclofenac at low dose (0.25 mg/kg) and high dose (2.5mg/kg), respectively, as compared to normal level of 3.3 to 5.3 mg/dl respectively in vultures.

Kidney plays an important role in elimination of uric acid and creatinine in birds. Blood flow to the avian kidney is different from blood flow in kidney of mammals. In mammalian kidney PGE2 and PGI2 function as renal vasodilators and regulate renal blood flow, supplied primarily through the afferent arteriole (Verlander, 1997). Diclofenac blocks PGE2 synthesis through inhibition of COX (Vinals *et al*., 1997). But in avian kidney, the renal portal system via the afferent renal portal vein is the primary nutrient blood source for the renal cortex and does not supply the renal medulla and medullary cone (Braun, 1993; Smith *et al*., 2000). The primary blood supply to avian glomeruli and DCT is central artery. Blood is supplied to the PCT via renal portal system. The effect of diclofenac on this system may be responsible for higher sensitivity of birds to its renal toxicity than mammals.

Meteyer *et al*. (2005) proposed a mechanism by which diclofenac induced renal failure through the inhibition of modulating effect of prostaglandin on angiotensin II mediated adrenergic stimulation. Presence of functional angiotensin system has been documented in birds (Goldstein and Skadhaughe, 2000). Renal portal valve opens in response to adrenergic stimulation, redirecting portal blood to the caudal venacava bypassing the kidney. Therefore, interference in prostaglandin modulation on renal portal valves by diclofenac might be resulting in indiscriminate action of the valves redirecting the primary nutrient blood supply away from the renal cortex leading to ischemic necrosis of PCT.

The preferential necrosis of PCT caused by diclofenac may also be related to the high metabolic activity of cells making them more sensitive to hypoxia than the cells in the DCT and CT, that are less metabolically active (Braun, 1985). The direct effect of diclofenac on mitochondria, resulting in compromised ATP synthesis and cytotoxic effect by a metabolite of diclofenac also need to be considered (Bort *et al*., 1999). Because uric acid excretion occurs at PCT and is an energy dependent process (Siller, 1981; Goldstein and Skadhaughe, 2000), decreased ATP either by hypoxia or direct cytotoxicity would contribute to hyperuricaemia.

PCT are also the primary site of uric acid excretion and reabsorption of ultrafiltrate (Henderson *et al*, 1993). Necrosis of PCT would compromise uric acid excretion, leading to rapid elevation of uric acid concentration in blood. Once the saturation point is reached in the blood, uric acid would rapidly precipitate as crystals on organ surface and within organ parenchyma resulting in death. The increased uric acid level observed in this study, following diclofenac administration might be due to renal failure which lead to hyperuriceamia and gout.

Diclofenac affects kidney of various genera of birds differentially. The possibility of differential physiologic response to diclofenac may also play a role in differential toxicity. The regulatory paths of nutrient blood supply to renal cortex differ between species (Goldstein and Skadhaughe, 2000) and the sensitivity of renal portal valve to diclofenac may also vary between species. There is species variation in the anatomy of renal portal system and species anatomy of these structure is still unknown (Johnson, 1979).

The mechanism of diclofenac induced renal toxicity in broiler birds is unknown, but the roles of PGE2 and PGI2 and COX I in smooth muscle control of renal portal valve and its modification by diclofenac may be the cause of renal

damage observed in present study. In the present investigation broiler birds found to be less sensitive compared to toxicity in vultures, reported by several investigators (Swan et al., 2005; Meteyer, et al., 2005).

Creatinine is NPN waste found in the blood and formed in the metabolism of muscle creatine and phosphocreatine. Creatinine after being filtered by glomerulus is excreted in urine. Since it is not excreted or absorbed by renal tubules to any degree it can be used as rough index of GFR (Benjamin, 1985). Increased level of plasma creatinine observed in this study following administration of diclofenac sodium in broiler birds might be related to blockade of renal vasodilatation (Verlander, 1997) due to nonseletive inhibition of the cyclooxygenase by diclofenac sodium.

Effect on Plasma Glutamic Pyruvic Transaminase

Table 12 and Fig. 7 show the mean values of plasma glutamic pyruvic transaminase (GPT) activity in the three groups at different interval of the single dose study. The GPT activity in the control group (I), at pre- and post-treatment intervals ranged between 8.01 ± 0.84 and 9.3 ± 0.58 U/L and was statistically similar. In the low dose group (II) the enzyme activity at pre and post- treatment intervals ranged between 7.02 ± 0.41 and 18.18 ± 1.5 U/L, where the enzyme activity at 12 hr (18.18 ± 1.56 U/L) and 24 hr (11.93 ± 0.71 U/L) post treatment was significantly higher than that of control group as well as that of pre-treatment and 36 hr post-treatment. In the high dose group (III), the GPT levels in the surviving birds were also statistically higher at 12 hr (21.83 ± 1.43 U/L) and 24 hr (14.33 ± 0.51 U/L) post treatment as compared to pre-treatment and 36 hr post treatment. The GPT activity at 12 hr, both in group II and live birds of Group III were significantly much higher than the control whereas the GPT activity was significantly higher at 24 hr than 12 hr in birds of Group III died 24 hr post treatment. At 36 hr post-treatment the GPT activity again returned

to normal control value in the birds of Group II and surviving birds of Group III. The birds which died after 24 hr in the high dose group, showed significantly elevated GPT activity at 12 and 24 hr post treatment (28.33 ± 0.84 and 37.83 ± 0.96 U/L) than the control and these values were also significantly higher than GPT levels of surviving birds (GroupIII) and Low dose group (II).

Table 12: Effect of single dose oral administration of diclofenac sodium on plasma glutamic pyruvic transaminase (PGPT) in broiler birds

Group No.	Dose of Diclofenac sodium		Mean Plasma Glutamic Pyruvic Transaminase (PGPT) (U/L) ± SE			
			Pre-treatment (0 hr)	Post-treatment (hr)		
				12	24	36
I	Control		$8.01^{a,d}$ ± 0.84 (6)	8.7^{a} ± 0.79 (6)	$9.1^{a,e}$ ± 0.72 (6)	$9.3^{a,g}$ ± 0.58 (6)
II	2 mg/kg		$8.01^{b,d}$ ± 0.84 (6)	18.18 ± 1.56 (6)	$11.93^{e,f}$ ± 0.71 (6)	$7.02^{b,g}$ ± 0.41 (6)
III	20 mg/kg	Live birds	$8.27^{c,d}$ ± 1.07 (3)	21.83 ±1.43 (3)	14.33^{f} ± 0.51 (3)	$7.5^{c,g}$ ± 0.39 (3)
		Dead birds	7.76^{d} ± 0.75 (3)	28.33 ± 0.84 (3)	37.83 ± 0.96 (3)	Died

Figures in parentheses refer to no. of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

Fig. 7: Plasma glutamic pyruvic transaminase levels of broiler birds treated with single oral dose of diclofenac sodium

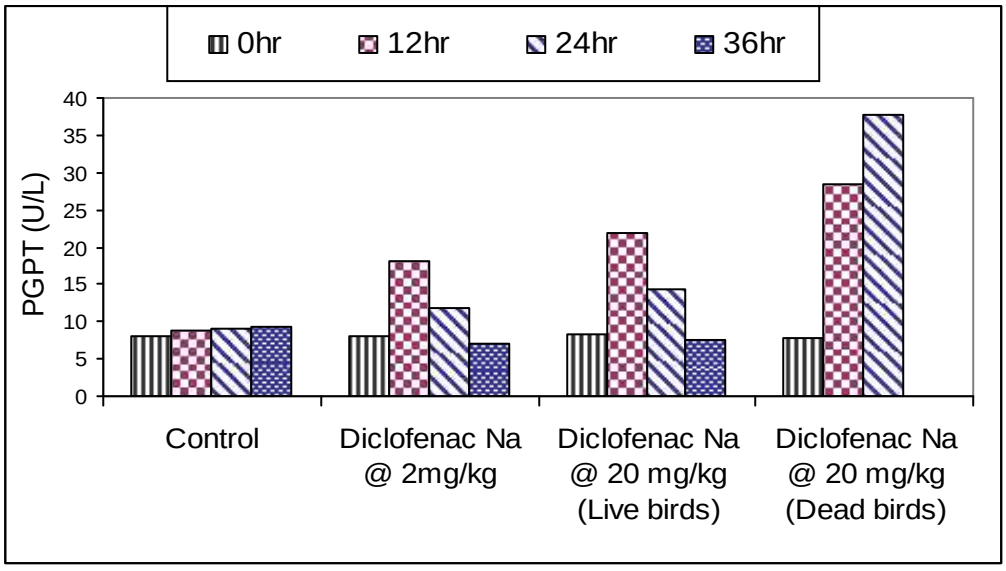


Table 13 shows the effect of repeated oral administration of diclofenac sodium @ 2mg/kg/day for 14 days in broiler birds. The enzyme activity during pre-treatment and post-treatment among the diclofenac treated birds (8.94 ± 0.65 and 9.19 ± 0.72) were statistically similar to the enzyme activity among the control birds (9.10 ± 0.65 and 8.66 ± 0.35).

Table 13: Effect of repeated oral administration of diclofenac sodium on plasma glutamic pyruvic transaminase (PGPT) levels in broiler birds

Group No.	Treatment	Mean PGPT (U/L) ± SE	
		Pre-treatment	Post-treatment
I	Control	9.10 ± 0.65 (6)	8.66 ± 0.36 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	8.94 ± 0.65 (6)	9.19 ± 0.72 (6)

Figures in parentheses refer to no. of observations

In the present study, the elevated plasma GPT activity by diclofenac sodium administration in broiler birds is suggestive of hepatic damage (Lumeij, 1999 ; Denman *et al*., 1983). The decrease in plasma protein, albumin and histopathological changes in liver section, support the above findings. The hepatotoxic effect of various NSAIDs (including diclofenac) and its mechanism of action have been reported by several workers. Diclofenac can block the activity of the adenine nucleotide translocase and inhibit mitochondrial ATPase activity (Moreno-Sanchez *et al*., 1999).

The association of NSAIDs with liver disease is poorly documented. Reports on hepatic injury have ranged from insignificant and transient liver enzyme elevation to severe fulminant hepatitis (Monukian *et al*., 1996).

Diclofenac causes a rare but potentially fatal hepatotoxicity that may be associated with the formation of reactive metabolites (Kappus, 1968). These products presumably were formed via a hepatic cytochrome P450 catalyzed oxidation of diclofenac to reactive benzoquinones imines that are trapped by GSH (glutathione) conjugation. The benzoquinones imines so formed contribute to diclofenac mediated hepatic injury (Tang *et al*., 1999).

HEMATOLOGICAL PROFILE

The effect of repeated oral administration of diclofenac sodium (2 mg/kg/day for 14 days) on hematology (haemoglobin, total erythrocyte count, total leucocyte count, differential leucocyte count and clotting time) of broiler birds was investigated.

Effect on Hemoglobin

Table 14 gives the mean hemoglobin levels of broiler birds in control group (I) and those treated with diclofenac sodium (Group II). The hemoglobin levels at pre- as well as post-treatment in the control group (9.65 ± 0.36 to 9.65 ± 0.33 g %) and diclofenac sodium treated group (8.66 ± 0.20 to 9.06 ± 0.56 g %) were statistically similar.

Table 14: Effect of repeated oral administration of diclofenac sodium on hemoglobin levels in broiler birds

Group No.	Treatment	Mean Blood Hemoglobin (g%) ± SE	
		Pre-treatment	Post-treatment
I	Control	9.60 ± 0.33 (6)	9.65 ± 0.36 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	9.06 ± 0.56 (6)	8.66 ± 0.20 (6)

Figures in parentheses refer to number of observations

Effect on Total Erythrocyte Count

Table 15 summarizes the effect of oral administration of diclofenac sodium on total erytrhocyte count in broiler birds. There were no significant differencesin TEC in the control group at pre- and post-treatment level (3.46 ± 0.23 to 3.46 ± 0.25 millions/µl). Statistical similarity was also found in treated group at respective levels (4.05 ± 0.31 and 3.58 ± 0.20 millions/µl).

Table 15: Effect of repeated oral administration of diclofenac sodium on total erythrocyte count (TEC) in broiler birds

Group No.	Treatment	Mean TEC (mollion / µl) ± SE	
		Pre-treatment	Post-treatment
I	Control	3.46 ± 0.23 (6)	3.46 ± 0.25 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	4.05 ± 0.31 (6)	3.58 ± 0.20 (6)

Figures in parentheses refer to number of observations

Effect on Total Leukocyte Count

Table 16 describes the effect of oral administration of diclofenac sodium on total leukocyte count in broiler birds. The count in control group and that of diclofenac treated group at pre-treatment (14.96 ± 5.77 and 16.65 ± 9.42 thousands/µl respectively) and at post-treatment were statistically similar (16.33 ± 8.22 and 16.03 ± 7.39 thousands/µl) were statistically similar.

Table 16: Effect of repeated oral administration of diclofenac sodium on total leukocytes count (TLC) in broiler birds

Group No.	Treatment	Mean TLC (thousands / μl) ± SE	
		Pre-treatment	Post-treatment
I	Control	14.96 ± 5.77 (6)	16.33 ± 8.22 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	16.65 ± 9.42 (6)	16.03 ± 7.39 (6)

Figures in parentheses refer to number of observations

Effect on Differential Leukocyte Count

The data of differential leukocyte count (DLC) of Groups I and II are given in Table 17.

The mean lymphocyte count during post-treatment in two groups varied between 60.66 ± 2.20 and 64.16 ± 0.87 % as compared to the pre-treatment count which ranged from 61.61 ± 1.16 to 61.50 ± 1.30 %. The variation in lymphocyte count within and between groups was insignificant at pre- and post-treatment period.

The mean heterophil count during post-treatment in two groups varied between 24.33 ± 0.84 and 27.0 ± 0.85 % as compared to the pre-treatment count which ranged from 25.66 ± 1.22 to 26.66 ± 1.16 %. The variation in hetrophil count within and between groups was insignificant at pre- and post-treatment period.

The mean monocyte count during post-treatment in two groups varied between 5.50 ± 0.22 and 6.5 ± 0.42 % as compared to the pre-treatment count which ranged from 5.66 ± 0.21 to 5.83 ± .33 %. The variation in monocyte count within and between groups was insignificant at pre- and post-treatment period.

The mean eosinophil count during post-treatment in two groups varied between 4.83 ± 0.47 and 5.33 ± 0.33 % as compared to the pre-treatment count which

ranged from 4.33 ± 0.33 to 4.66 ± 0.21 %. The variation in eosinophil count within and between groups was insignificant at pre- and post-treatment period

The mean basophil count during post-treatment in two groups varied between 0.0 and 0.16 % as compared to the pre-treatment count which ranged from 0.0 to 0.33 %. The variation in basophil count within and between groups was insignificant at pre- and post-treatment period.

Effect on Clotting Time

The effect of repeated oral administration of diclofenac sodium on clotting time is explained through Table 18 and Fig. 8. The mean clotting time in the control group during pre- and post-treatment varied between 2.76 ± 0.20 and 2.83 ± 0.16 minutes and the variation was insignificant. The pre-treatment clotting time in Group II (2.61 ± 0.09 minute) was also statistically similar to that of control group. However, it was significantly higher on 15th day of post-treatment (6.7 ± 0.37 minute) as compared to its pre-treatment as well that of post-treatment clotting time of control group.

Table 18: Effect of repeated oral administration of diclofenac sodium on clotting time in broiler birds

Group No.	Treatment	Mean Clotting Time (minutes) ± SE	
		Pre-treatment	Post-treatment
I	Control	2.83^{a,b} ± 0.16 (6)	2.76^{a} ± 0.20 (6)
II	Diclofenac sodium @2 mg/kg/day for 14 days	2.61^{b} ± 0.09 (6)	6.7 ± 0.37 (6)

Figures in parentheses refer to no. of observations
Mean values with similar superscripts within the rows and columns are statistically similar (P > 0.05)

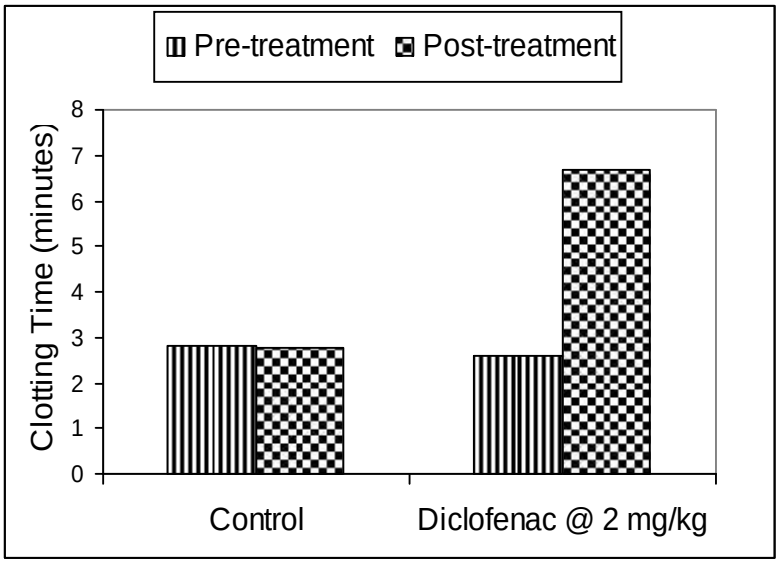
Table 17: Effect of Repeated oral administration of Diclofenac sodium on Differential Leucocyte Count (DLC) in broiler birds

Group No.	Diclofenac sodium	Mean DLC (%) ± S.E.									
		Pre-treatment (0day)					Post-treatment (14day)				
		H	L	M	E	B	H	L	M	E	B
I	Control	25.66 ± 1.22	61.16 ± 1.16	5.83 ± 0.33	4.33 ± 0.33	0	26.16 ± 1.16	61.5 ± 1.3	5.66 ± 0.21	4.66 ± 0.21	0.33 ± 0.21
II	2 mg/kg/day for 14 days	24.33 ± 0.84	64.16 ± 0.87	5.5 ± 0.22	6.0 ± 0.40	0	32.83 ± 4.49	55.66 ± 0.47	6.5 ± 0.47	4.83 ± 0.47	0.16 ± 0.16

Figs. in parentheses refers to no.of observations

H: Heterophils; L: Lymphocytes; M: Monocytes; E: Eosionophils; B: Basophils

Fig. 8: Clotting Time of broiler birds treated with repeated oral dose of diclofenac sodium (2 mg/kg/day)



Repeated dosing of diclofenac @ 2 mg/kg/day for 14 consecutive days significantly prolonged the blood clotting time in broiler birds and this might be related to COX inhibition. It is well known that most of the NSAIDs delay blood clotting through cyclooxygenase inactivation (both COX I and COX II) and consequent inhibition of synthesis of thromboxanes, which are essential for platelet aggregation and blood clotting mechanisms (Rang *et al*., 2003).

PATHOLOGICAL CHANGES

All the birds which died due to toxicity of diclofenac in single high dose group experiments, were autopsied and subjected to pathological observations. Similarly the surviving birds in both the toxicity studies were also sacrificed and autopsied for pathological investigations.

Gross Pathology

There was no mortality among the birds treated with single low dose of diclofenac (2mg/kg, po). The birds of this group which were sacrificed did not show any gross pathological changes in the visceral organs. However, the birds died following

administration of single high dose of the drug (20 mg/kg, po) on post mortem, showed visceral gout characterized by chalky white deposition on the serosal surface of visceral organs (Fig.10) as compared to the normal viscera of the control bird (Fig.9) which revealed no gout. The chalky white deposit on the serosal surface was confirmed to be uric acid by Murexide test (Sharma, 1997).

The three birds which succumbed to diclofenac sodium toxicity at 20mg/kg (single high dose) also showed sporadic congestion all through the length of intestine. The survived three birds which were sacrificed did not show any gross lesions except sporadic congestion in the intestine.

Similar gross lesions of visceral gout has been reported by Oak's *et al*. (2004), Arun and Azeez (2004) and Shultz *et al.* (2004) in vultures and Patel *et al.* (2007) in chicks died due to diclofenac toxicity. Similar observations were also reported by several other investigators (Damodaran *et al*., 1978; Chandra *et al*., 1980; Nayak *et al*., 1988; Rao *et al.*, 1993; Uma *et al.*, 1999) in birds.

Histopathology

Liver: The normal histostructure of liver of the control is depicted in (Fig. 11). The liver section of broiler birds following diclofenac sodium treatment (@ 2mg/kg, single low dose, po) showed mild hydropic degeneration (Fig.12). Liver section of sacrificed broiler bird which remained alive following diclofenac treatment (@ 20 mg/kg, single high dose, po) exhibited periportal fibrosis (Fig.13) and focal aggregation of lymphocytes (Fig. 14). Liver section of broiler birds which succumbed to diclofenac sodium toxicity following administration of single high dose also showed severe necrosis with infiltration of mononuclear cells (Fig.15). Aydin *et al*. (2002) reported similar changes in liver tissue induced by diclofenac sodium in rats.

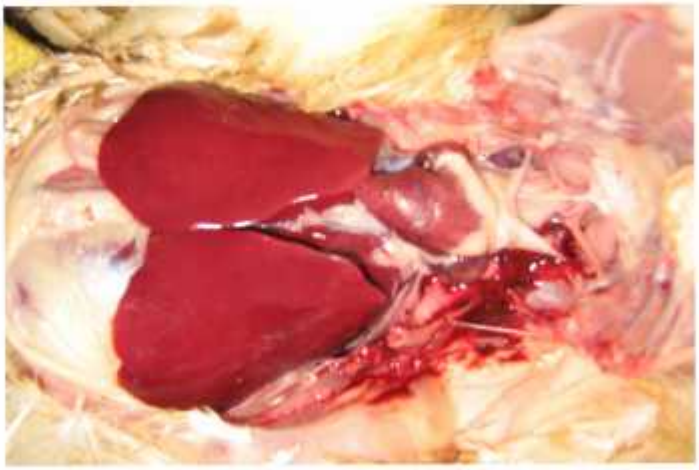


Fig.9: Viscera of control broiler bird

Fig.10: Viscera of broiler bird treated with diclofenac-Na (@ 20 mg/kg, po) showing chalky deposition of uric acid

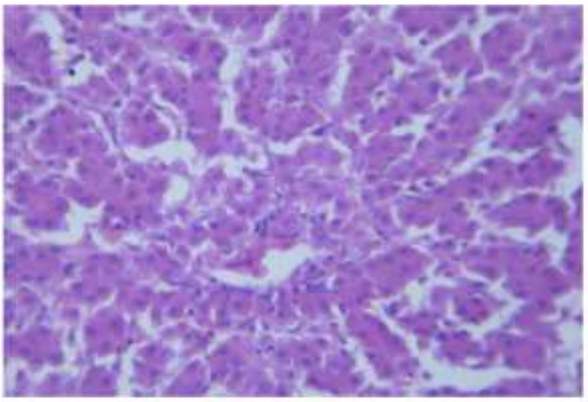


Fig.11: Liver section (H&E X 400) of control broiler bird showing normal histoarchitecture

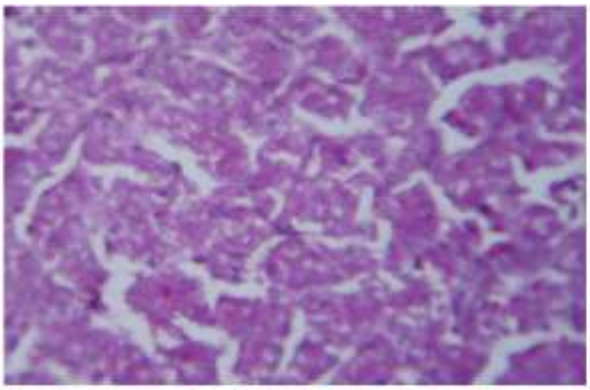


Fig.12: Liver section (H&E X 400) of broiler bird following diclofenac-Na treatment (@ 2 mg/kg, po) showing hydropic degeneration

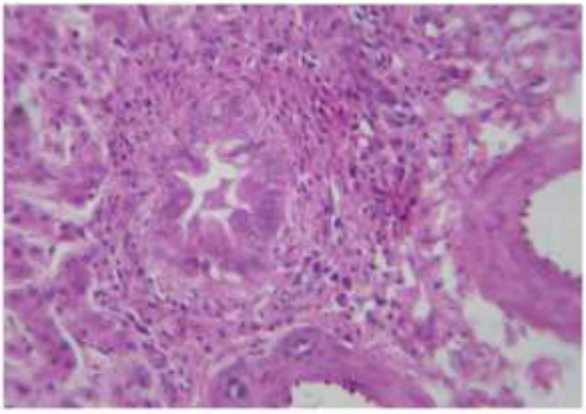


Fig.13: Liver section (H&E X 400) of sacrificed broiler bird following diclofenac-Natreatment (@ 20 mg/kg, po) showing fibrous tissue proliferation in portal area

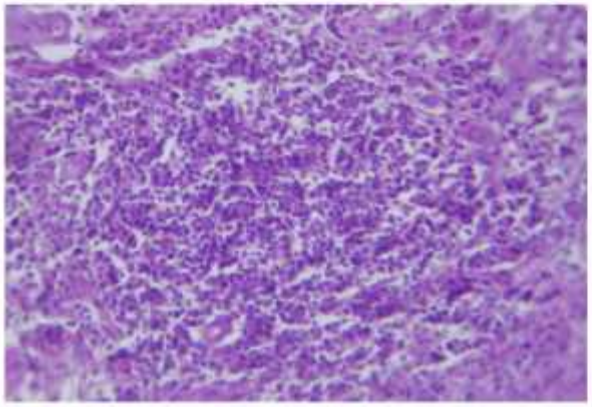


Fig.14: Liver section (H&E X 400) of sacrificed broiler bird following diclofenac-Na treatment (@ 20 mg/kg, po) showing focal aggregation of lymphocytes

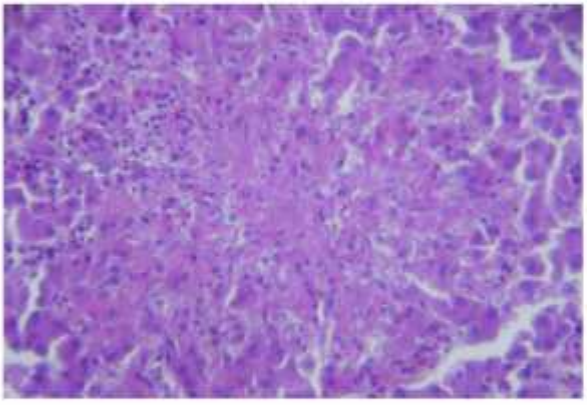


Fig.15: Liver section (H&E X 400) of broiler bird succumbed to diclofenac-Na toxicity (@ 20 mg/kg, po) showing severe necrosis with mononuclear cell infiltration

The lesions observed during the present study were similar to those reported by Uma *et al.* (1999) and Rao, *et al.* (1993) during their study on gout in birds. The liver sections of repeated dose group (2 mg/kg/day, po) did not reveal any altered histoarchitecture. The histopathological examination of liver section indicated liver damaging effect of diclofenac sodium in broiler bird particularly at higher dose.

Kidney: Fig.16 shows the section of kidney of control broiler bird having normal histoarchitecture. Kidney section of broiler birds following diclofenac sodium treatment (@ 2 mg/kg, single low dose, po) showed mild degenerative changes in the renal tubules.

Kidney section of sacrificed broiler birds which remained alive following diclofenac treatment at single high dose (@ 20 mg/kg, po) showed mild degenerative and necrotic changes in the renal tubules along with infiltration of mononuclear cells in the intertubular areas. The kidney section of birds which succumbed to diclofenac sodium toxicity at single high dose (@ 20 mg/kg,po) showed tubular degeneration and varying sized foci of urate deposition (tophi) either in form of amorphous material or in radiating crystalline pattern mixed with necrotic debri due to degeneration of surrounding cells (Fig.17).

Oaks *et al.* (2004) found similar findings in all the OWBVs died due to diclofenac toxicity. Similar findings were also reported by Meteyer *et al.* (2005) in kidney section of 55 OWBVs and several others in poultry (Damodaran *et al*., 1978; Chandra *et al*., 1980; Nayak *et al*., 1988; Rao *et al.*, 1993; Uma *et al.*, 1999; Patel *et al*., 2007) in case of gout in birds.

The kidney section of broiler bird following repeated diclofenac sodium treatment @ 2 mg/kg/day for 14 days showed mild degenerative changes with albuminous casts in the renal tubules (Fig.18).

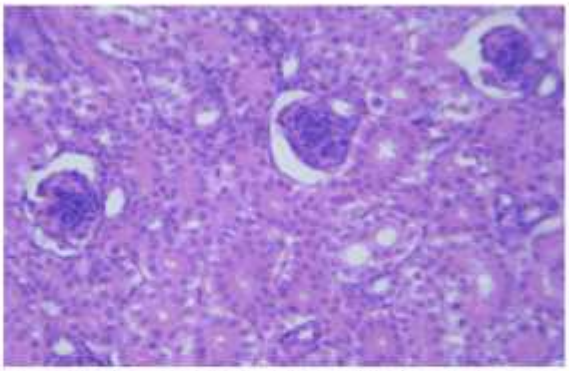


Fig.16: Kidney section (H&E X 400) of control broiler bird showing normal histoarchitecture

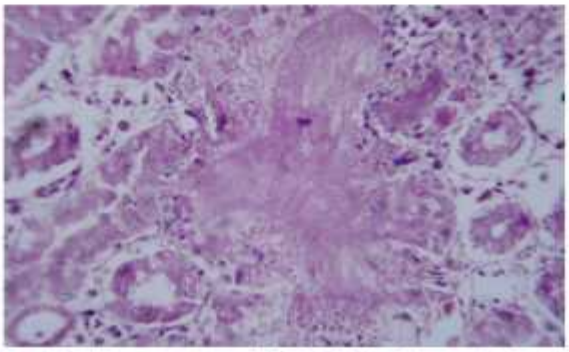


Fig.17: Kidney section (H&E X 400) of broiler bird succumbed to diclofenac-Na toxicity (@ 20 mg/kg, po) showing radiating uric acid crystal leading to degeneration of surrounding cells

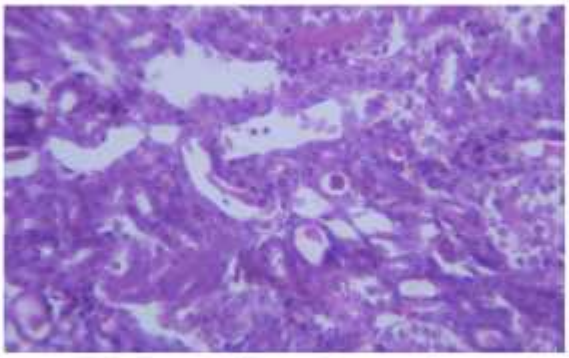


Fig.18: Kidney section (H&E X 400) of broiler bird following diclofenac-Na treatment (@ 2 mg/kg/day for 14 days) showing mild degenerative changes with albuminous cast

In this study, the histopathological changes observed in the kidney section along with changes in plasma uric acid and creatinine were suggestive of renal damage.

Heart: Fig. 19 shows the section heart of control broiler bird having normal histoarchitecture. The heart section of broiler bird following diclofenac sodium treatment @ 2 mg/kg (single low) and live birds of high dose (20 mg/kg, po) did not show any histopathological changes. While heart section of broiler birds which succumbed to diclofenac sodium toxicity (single high dose, @ 20 mg/kg), po) showed necrosis of muscle fibre, oedema and thickening of epicardium which might be due to presence of acellular eosinophilic material (Fig.20 and 21).

Similar lesions in heart were also reported by Uma *et al*. (1999) during their study on pathology of gout in poultry. Heart section of birds of repeated dose group did not show any change.

Mitochondrial damage or production of highly reactive diclofenac metabolites as discussed in hepatotoxicity (Farell, 1997) may be responsible for diclofenac induced cardiotoxicity.

Spleen: Spleen sections of boiler birds did not show any marked changes in birds of single low dose group. The section of spleen of survived and sacrificed birds of single high dose (@ 20 mg/kg, po) of diclofenac sodium also did not show any altered histoarchitecture. But the section of broiler birds which succumbed to diclofenac sodium toxicity showed depletion of lymphocytes from the white pulp of spleen and radiating necrotic mass indicating area deposited with urate crystals. The spleen section of birds of repeated dose group did not show any histopathologic changes.

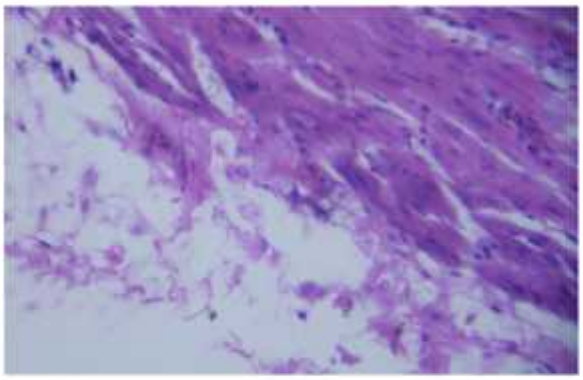


Fig.19: Heart section (H&E X 400) of control broiler bird showing normal histoarchitecture

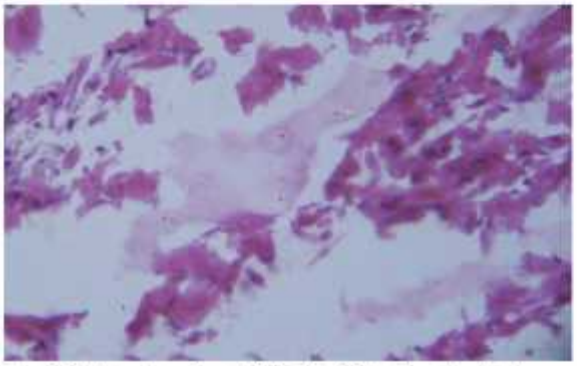


Fig.20: Heart section (H&E X 400) of broiler bird succumbed to diclofenac-Na toxicity (@ 20 mg/kg, po) showing oedema and necrosis of cardiac muscle

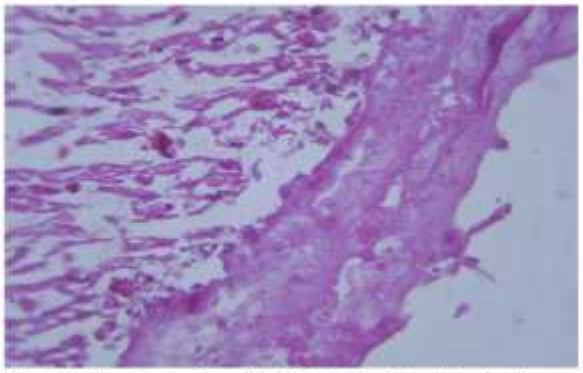


Fig.21: Heart section (H&E X 400) of broiler bird succumbed to diclofenac-Na toxicity (@ 20 mg/kg, po) showing thickening of epicardium

Intestine: The section of intestine of boiler birds did not show any histopathological change in the single low dose (2 mg/kg of diclofenac sodium) group, sacrificed bird of high dose (20 mg/kg of diclofenac sodium) group and repeated dose group (2 mg/kg/day of diclofenac sodium for 14 days). Congestion was only seen in the mucosal layer of intestine in died birds of high dose group.

CHAPTER VI

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH WORK

Diclofenac sodium, a nonsteroidal antiinflammatory drug (NSAID) owes its pharmacological and adverse effects to preferential inhibition of COX I enzyme. Its widespread use in animal health has been linked to near extinction of vultures in the Indian subcontinent. The etiopathology of diclofenac-induced visceral gout in vultures is not conclusively understood. This has necessitated detailed studies of diclofenac in the genesis of visceral gout in avian species. Hence, the present investigation was undertaken to evaluate the toxicity of diclofenac sodium in broiler birds following oral exposure to single and repeated doses.

Apparently healthy adult broiler birds (Vencob) of about 6 weeks of age were selected for the study. All the birds were kept in the deep litter system under hygienic conditions with free access to balanced feed and drinking water. After a seven-day acclimatization period to the experimental conditions the birds were randomly assigned to different groups for conduct of toxicity investigations.

The test drug, diclofenac sodium (Voveran®, 50 mg tablets) was evaluated for its toxicity by oral administration at single low dose (2 mg/kg, equivalent to mammalian clinical dose) and high dose (20 mg/kg) and repeated dosing of the low dose for 14 consecutive days (2 mg/kg/day).

In single dose study, 18 broiler birds were assigned to three groups of six birds in each. The first group (I) birds received only distilled water orally and served as control. The remaining two groups (II and III) orally received a single dose of diclofenac sodium in distilled water @ 2 mg/kg and 20 mg/kg respectively. Single dose toxicity was assessed by observing clinical signs, alterations in blood biochemistry and pathological changes. For repeated dose toxicity trial, 12 broiler birds were divided at random into

two groups of six birds in each. The first group was kept as control and received only distilled water orally. The other group (II) received diclofenac sodium orally @ 2mg/kg/day, for 14 consecutive days. The repeated dose toxicity was evaluated by observing toxicity signs and symptoms, change in body weight, alterations in biochemical and hematological profiles and pathological changes.

Following single low dose oral administration of diclofenac sodium @ 2 mg/kg, the broiler birds showed loose fecal droppings with no other adverse reaction or mortality up to seven days of post treatment. In single high dose study, 50% of birds treated with diclofenac sodium (@ 20 mg/kg, po) succumbed to toxicity within 36 hr post treatment and showed blood tinged diarrhoea, segregatory behaviour, dullness, lethargy and refusal of feed and water. The birds which survived following single administration of 20 mg/kg dose, showed only blood tinged fecal droppings. The birds treated with repeated low doses of diclofenac sodium (@ 2 mg/kg/day for 14 days) exhibited mild diarrhoea, with no other visible toxic manifestation or mortality up to 14 days.

Following single dose administration of diclofenac sodium @ 2 mg/kg and 20 mg/kg, po, the birds showed significant increase in plasma creatinine, uric acid and GPT levels and reduction in plasma total protein and albumin levels at 12 and 24 hr post treatment, where the alterations in general were directly related to the dose of diclofenc sodium.

The birds treated with single low dose of diclofenac sodium (@ 2 mg/kg, po) showed significantly lower total plasma protein at 12 hr and (1.93 ± 0.08 g/dl) and 24hr (2.68 ± 0.20 g/dl) of post–treatment compared to control birds. The plasma albumin also significantly decreased at 12 (1.64 ± 0.08 g/dl) and 24 hr (1.97± 0.02 g/dl) of post-treatment. The uric acid, creatinine and PGPT levels were significantly elevated at 12 hr (19.81 ± 0.41mg/dl, 0.90 ± 0.03mg/dl and 18.18 ± 1.56 U/L, respectively) and 24 hr

(11.03 ± 0.99 mg/dl, 0.73 ± 0.01 mg/dl and 11.93 ± 0.71 U/L, respectively) of post-treatment.

The surviving birds treated with single high dose of diclofenac sodium (@ 20 mg/kg, po) showed significantly lower total plasma protein at 12 and (1.66 ± 0.02 g/dl) and 24hr (3.11 ± 0.06 g/dl) of post–treatment compared to control birds. The plasma albumin also significantly decreased at 12hr (1.29 ± 0.08 g/dl) and 24 hr (2.33± 0.17 g/dl) post-treatment. The uric acid, creatinine and PGPT levels were significantly elevated at 12 hr (24.50 ± 0.73 mg/dl, 1.08 ± 0.04 mg/dl and 21.83 ± 1.43 U/L, respectively) and 24 hr (12.60 ± 0.49 mg/dl, 0.99 ± 0.00 mg/dl and 14.33 ± 0.51 U/L, respectively) of post-treatment.

All the above parameters, in general, had a tendency to return back to the control levels at 36 hr post- treatment in the birds treated with single low dose of diclofenac and the surviving birds treated with high dose of diclofenac sodium.

The birds which succumbed following treatment with single high dose of diclofenac sodium (@ 20 mg/kg, po) showed significantly lower total plasma protein at 12 and (1.66 ± 0.08 g/dl) and 24hr (1.07 ± 0.03 g/dl) of post–treatment as compared to control birds. The plasma albumin also significantly decreased at 12hr (1.01 ± 0.01 g/dl) and 24 hr (0.91 ± 0.03 g/dl) of post-treatment. The uric acid, creatinine and PGPT levels were significantly elevated at 12 hr (26.43 ± 0.93mg/dl, 1.35 ± 0.12 mg/dl and 28.33 ± 0.84 U/L, respectively) and 24 hr (39.93 ± 1.79 mg/dl, 1.56 ± 0.03 mg/dl and 37.83 ± 0.96 U/L, respectively) of post-treatment.

Repeated dosing of diclofenac sodium @ 2 mg/kg/day for consecutive 14 days also showed significant increase in uric acid (6.97 ± 0.32 mg/dl) and creatinine (0.73 ± 0.04 mg/dl) levels. There were no alterations in body weight gain and hematological

profile but significant prolongation of clotting time (6.70 ± 0.37 min) was observed at the end of the experiment.

The birds died following treatment with single high dose (20 mg/kg, po) of diclofenac sodium, on post-mortem showed extensive visceral gout characterized by chalky white deposition on the serosal surface of visceral organs. The birds of control group and low dose group did not show any gross pathological lesions. The surviving birds of high dose group showed only sporadic congestion in the intestine.

The histopathological observation revealed mild hydropic degeneration in liver and mild degenerative changes in renal tubules in the birds treated with single low dose (@ 2 mg/kg, po) of diclofenac sodium. The birds died following treatment with single high dose (@20 mg/kg, po) revealed aggressive lesions, characterized by severe necrotic changes with infiltration of heterophils in portal areas; degenerative and necrotic changes, presence of albuminous casts and urate deposits (tophi) with infiltration of mononuclear cells in renal tubules; oedema, congestion and hyalinization of cardiac muscle and lymphocyte depletion from white pulp and presence of tophi in spleen. The birds which sustained the high dose showed proliferation of fibrous tissue in portal areas and lymphoid foci in liver parenchyma and moderate degenerative changes in kidney. The birds treated with repeated doses of diclofenac sodium (@ 2 mg/kg/day for 14 days) did not reveal any prominent histopathological changes except mild degenerative changes with presence of albuminous material in distal convoluted tubules in kidney.

From the present investigation it may be concluded that diclofenac sodium has high hepatotoxic and nephrotoxic potential inducing visceral gout in broiler birds.

Conclusions

1. Diclofenac sodium at a single oral dose of 2 mg/kg corresponding to the mammalian therapeutic/clinical dose was non-lethal but mildly toxic to broiler birds.

2. Diclofenac sodium at ten times the mammalian clinical dose (@ 20 mg/kg, po) was highly toxic to the broiler birds causing 50 % fatality with extensive visceral

gout, significant reduction in plasma protein and albumin and high elevation in uric acid, creatinine and plasma glutamic pyruvic transaminase.

3. Repeated oral administration of diclofenac sodium @ 2 mg/kg/day for 14 days was also non-lethal, but caused mild renal impairment and prolonged the blood clotting time in broiler birds.

4. Diclofenac sodium, a preferential COX I inhibitor, has high hepatotoxic and nephrotoxic potential inducing visceral gout in broiler birds. .

5. Diclofenac sodium, a preferential COX I inhibitor, also interferes with the blood clotting cascade in poultry birds.

Suggestions for Future Research Work

1. A better understanding of the exposure of diclofenac to different species including birds and knowledge on the mechanism of NSAIDs toxicity are urgently needed, to enable a better assessment of the future impact of NSAIDs on the environment and to determine which NSAIDs can be safely used to replace diclofenac.

2. Because of the great differences in sensitivity between species, the pharmacology and toxicology of each NSAIDs should be independently assessed for each target species, including different species of birds.

3. The status of cyclooxygenase and its isoenzymes, and the role of prostanoids in poultry birds need to be investigated.

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ABSTRACT

Diclofenac sodium, a nonsteroidal antiinflammatory drug (NSAID) owes its pharmacological and adverse effects to preferential inhibition of COX I enzyme. Its widespread use in animal health has been linked to near extinction of vultures in the Indian subcontinent. The etiopathology of diclofenac-induced visceral gout in vultures is not conclusively understood. This has necessitated detailed studies of diclofenac in the genesis of visceral gout in avian species. Hence, the present investigation was undertaken to evaluate the toxicity of diclofenac sodium in broiler birds following oral exposure to single and repeated doses.

Apparently healthy adult broiler birds (Vencob) of about 6 weeks of age were selected for the study. All the birds were kept in the deep litter system under hygienic conditions with free access to balanced feed and drinking water. After a seven-day acclimatization period to the experimental conditions the birds were randomly assigned to different groups for conduct of toxicity investigations.

In single dose study, eighteen birds were divided into three groups of six birds in each. All the birds were fasted overnight and weighed individually just prior to administration of the test drug. The Group I birds received only distilled water (vehicle) orally and served as control. The birds in Groups II and III respectively received single oral dose of diclofenac sodium @ 2 mg/kg (corresponding to mammalian therapeutic dose) and 20 mg/kg (high dose) suspended in distilled water. Immediately after administration of the drug, the birds were observed for recording the time of onset, nature and severity of clinical symptoms and behavioral changes, and mortality, if any, up to 7^{th} day of post administration. In addition, blood samples from the birds in the three groups were also analysed for estimation of total protein, albumin, uric acid, creatinine and glutamic pyruvate transaminase (GPT) in plasma using Bayer's reagent kits at pretreatment and at 12, 24 and 36 hr of post treatment. All the surviving birds were

continued under observation for seven days and then sacrificed. The dead birds and those sacrificed were subjected to gross necropsy and histopathology of liver, kidney, heart, spleen and intestines.

For repeated dose study, 12 birds were divided into two groups, each consisting of six birds. The first group was kept as control and received only the distilled water orally. The other group birds were orally administered diclofenac sodium @ 2 mg/kg/day for consecutive 14 days. All the birds were constantly observed up to a period of 14 days for appearance of toxic manifestations and mortality, if any. Blood samples from the birds in both the groups were analysed for the biochemical profile as above at pre treatment and on 15^{th} day. In addition the total erythrocyte, leucocyte and differential leucocyte counts; hemoglobin and clotting time were also determined at pre and post treatments. At the end of the observation period all the treated birds were sacrificed for observing gross necropsy and histopathology as in single dose study.

Following single dose administration @ 2 mg/kg the broiler birds showed loose fecal droppings with no other adverse reaction up to seven days of post treatment. The birds treated with diclofenac sodium at 20 mg/kg, 50% succumbed to toxicity after 24 hr of post treatment and showed blood tinged diarrhoea, segregatory behaviour, dullness, lethargy and refusal of feed and water intake. The 50% birds which survived after the 20 mg/kg dose, only showed blood tinged fecal droppings. The birds repeatedly treated with diclofenac sodium (2 mg/kg/day) exhibited mild diarrhoea, with no other visible toxic manifestation or mortality up to 14 days.

Following single dose diclofenac sodium treatment at 2 mg/kg or 20 mg/kg the birds showed significant increase in plasma creatinine, uric acid and GPT levels and reduction in plasma total protein and albumin levels at 24 hr post treatment, where the alterations in general were directly related to the dose of diclofenac sodium. Repeated dosing of diclofenac sodium @ 2 mg/kg/day showed significant increase in creatinine and

uric acid levels and no alteration in hematological profile, except significantly prolonged clotting time at the end of the experiment.

The histopathological observation revealed mild hydropic degeneration in liver and mild degenerative changes in renal tubules among the birds treated with single dose (2 mg/kg, po) of diclofenac sodium. The dead birds following treatment with 20 mg/kg dose revealed severe necrotic changes with infiltration of heterophils in liver; degenerative and necrotic changes, presence of albuminous casts and urate deposits (tophi) with infiltration of mononuclear cells in renal tubules; oedema, congestion and hyalinization of cardiac muscle and lymphocyte depletion from white pulp and presence of tophi in spleen. The birds which sustained the high dose showed proliferation of fibrous tissue in portal areas and lymphoid foci in liver parenchyma and moderate degenerative changes in kidney. The birds treated with repeated doses of diclofenac sodium (2 mg/kg/day) did not reveal any prominent histopathological changes except mild degenerative changes with presence of albuminous material in distal convoluted tubules in kidney.

From the present investigation it may be concluded that diclofenac sodium has high hepatotoxic and nephrotoxic potential inducing visceral gout in broiler birds.